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(57) Abstract

This invention provides a method to induce MHC class II gene expression in a cell capable of MHC class II gene expression which comprises transfecting the cell with a retroviral vector encoding a CII-TA protein under suitable conditions so as to express the CII-TA protein and induce MHC class II gene expression. This invention also provides a method to stimulate a specific, high-level T-cell response in a subject, or in cells, in particular, wherein the cell is a neoplastic cell or a somatic cell including, a melanocyte, a hematopoietic cell, or a stem cell.

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RETROVIRUS VECTORS FOR EXPRESSION OF CII-TA AND ACTIVATION OF HLA CLASS II GENE EXPRESSION AND USES THEREOF

This application is a continuation-in-part of U.S. Serial No. 08/478,457, filed June 7, 1995, the contents of which are hereby incorporated by reference into the present application.

Background of the Invention

Major Histocompatibility Complex (MHC) class I and class II genes play an essential role in the immune response. The human MHC molecules, called HLA, are required by the immune system to activate responses against infectious pathogens (Bjorkman et al, 1987; Unanue, 1984). Early in an immune response, foreign antigens are internalized and processed by proteolytic digestion, and then returned to the cell surface in association with class II molecules. Interactions of a specific T cell receptor at the surface of the CD4+ T cell and antigenic peptide bound by a class II molecule on an antigen presenting cell (APC) lead to activation, proliferation, and/or differentiation of both T cells and APCs. The HLA class II region contains several alpha (A) and beta (B) genes (among them DP, DQ, and DR) located in a segment of several hundred kilobases (kb) on chromosome six, and these genes are expressed constitutively on relatively few tissues: among them B-cells, macrophages, and Langerhans-dendritic cells of the skin and lymphoid organs (Radka et al, 1986). Class II molecules are also expressed transiently along the developmental pathway of many hematopoietic cell types or following stimulation with various soluble factors, such as gamma interferon and IL-4 (Radka et al, 1986; Houghton et al, 1984; Noelle et al, 1984; Roehm et al, 1984). In general, B-cells express the DR, DP and DQ A and B genes coordinately suggesting that a common mechanism regulates their expression. Genetic evidence supporting this hypothesis has come from extensive studies of mutant B-cell lines generated in vitro or established from patients with class II deficient Bare

Lymphocyte Syndrome (Accolla, 1983; Calman and Peterlin, 1987; Touraine et al, 1984; Zegers et al, 1983). These cells show a concomitant decrease in expression of all class II molecules and in BLS patients the result is a lethal combined immunodeficiency (Lisowska Grospierre et al, 1985; Rijkers et al, 1987; de Preval et al, 1988; Plaeger-Marshal et al, 1988; Hume et al, 1989).

BLS is an autosomal recessive disease characterized by the absence of cellular and humoral T cell responses and impaired antibody production leaving patients susceptible to viral, bacterial, and fungal infections (Lisowska Grospierre et al, 1985). The infections usually begin in the first year of life and most often involve the respiratory and gastrointestinal systems. Clinically, patients exhibit malabsorption, failure to thrive and persistent, overwhelming viral infection. The prognosis of this disease remains dismal and even with supportive care very few patients survive into the second decade of life. The only curative therapy currently available is bone marrow transplant. Transplants however, can only be offered to the small number of patients who are fortunate to have a match. This and the other problems these patients face, such as failure to engraft and infectious complications, make another therapeutic option very desirable.

Summary of the Invention

This invention provides a method to induce MHC class II gene expression in a cell capable of MHC class II gene expression which comprises transfecting the cell with a retroviral vector encoding a CII-TA protein under suitable conditions so as to express the CII-TA protein and induce MHC class II gene expression. This invention also provides a method to stimulate a specific, high-level T-cell response in a subject, or in cells, in particular, wherein the cell is a neoplastic cell or a somatic cell including, a melanocyte, a hematopoietic cell, or a stem cell.

Brief Description of the Figures

Figure 1. CII-TA mutation resulting in lack of HLA class II expression in BLS-2 and BLS-2sib. Upper case = exon sequences, lower case = intron sequences.

Figure 2. Retrovirus constructs generated for transfection into producer cell lines.

The plasmid, pGAG-ßactin-CII-TA/S, was deposited on June 7, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid pGAG-ßactin-CII-TA/S was accorded ATCC Accession Number 97211. The plasmid, pGAG-Ii-CII-TA/S, was deposited on June 7, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid pGAG-Ii-CII-TA/S was accorded ATCC Accession Number 97212.

Figures 3A, 3B, 3C and 3D. Infection of BLS-2 with CII-TA retrovirus constructs. (3A) Uninfected. (3B) 3 days post-infection. (3C) 8 days post-infection. (3D) RAJ I (class II^+ B cell) as a positive control. Introduction of CII-TA into all of these cell line induces expression of HLA class II antigens, as shown by staining for HLA-DR.

Figures 4A, 4B, 4C and 4D. Infection of PBMCs from BLS patient with GAG-Ii/CII-TA virus. Cells were co-cultivated with PG13 virus producing cells and subsequently cultured for eight days in IL-2 containing media. DR expression was assessed in populations that were simultaneously stained for CD3 (T cells) and CD20 (B cells).

Figure 5. The 5′ LTR and the internal promoters in

GAG/ßactin-CIITA and pGAG/Ii-CIITA are active.

Figure 6. Growth of EBV lymphomas in SCID mice from transduced and untransduced cells.

Figures 7A, 7B, 7C and 7D. Selection for HLA class II expression in EBV lymphomas after growth in SCID mice.

Detailed Description of the Invention

This invention provides a method to induce MHC class II gene expression in a cell capable of MHC class II gene expression which comprises transfecting the cell with a retroviral vector encoding a CII-TA protein under suitable conditions so as to express the CII-TA protein and induce MHC Class II gene expression. The CII-TA cDNA sequence is published in Cell, 75: 135-146 (The contents of which are incorporated in their entirety into the subject application). Variants of CII-TA encoding the same amino acid sequence as in Steimle et al., 1993 are included in the subject application. Variants and mutants of CII-TA which are capable of homology hybridization under medium stringency or high stringency conditions are included in the subject application (for stringency conditions, see Kriegler, 1990). Functional variants with changes to the amino acid sequence with maintenance their biological activity are included in the subject application.

The retroviral vector may be a pGAG DNA retroviral vector, a N2 retroviral vector, a SIM retroviral vector, a LNL6 vector, a LXSN vector or a MMuLV retroviral vector (Anderson, 1992; Anderson et al., 1995; Rosenberg et al., 1990; Wilson et al., 1990; The contents of which are incorporated in their entirety into the subject application). The retroviral vector is replicative incompetent and has a suitably modified 3' long terminal repeat. The retroviral vector may encode a tissue specific promoter such as the HLA class II associated invariant chain promoter. The retroviral vector may include DNA in a 3' to 5' direction encoding the reverse transcript of at least a portion of a retrovirus, including a 3' long terminal repeat sequence, a promoter sequence, a sequence encoding a CII-TA protein and a 5' long terminal repeat sequence.

The most preferred condition for transfecting the target

cell is co-cultivating the target cell with a virus packaging cell which is infected with a retroviral vector encoding a CII-TA protein. The virus packaging cell line may be ψCRIP, ψCRE, AM12, E86, PA317 or PG13 (Jolly et al., 1992; Miller, 1990; The contents of which are incorporated in their entirety into the subject application). The induction of MHC class II gene expression in a cell may be increased by the co-transduction of a suitable immunoenhancer or adjuvant such as B7-1 protein or B7-2 protein or B-17 protein. The adjuvant may be transfected into the cell as part of a retroviral vector encoding the adjuvant. Alternatively, the adjuvant may be introduced into the cell by co-transduction which includes linking a sequence encoding the adjuvant to the retroviral vector encoding the CII-TA protein. The target cell may be a neoplastic cell or a somatic cell including, a melanocyte, a hematopoietic cell, a stem cell, or a BLS-2 cell.

One embodiment of this invention is a method to stimulate a specific, high-level T-cell response in a subject, which entails obtaining cells capable of MHC class II gene expression from the subject and transfecting them with a retroviral vector encoding CII-TA under suitable conditions so as to express CII-TA protein and induce MHC class II gene expression in the cells. The cells would then be tested for the presence of helper virus and other contaminants followed by the administration of clean cells to the subject to thereby induce expression of the class II human leukocyte antigen gene complex in the subject and thus stimulate the specific, high-level T-cell response in the subject. This method would be useful for a subject with Bare Lymphocyte Syndrome or a neoplastic condition. The cell obtained from the subject may be a neoplastic cell, a somatic cell, a melanocyte, a hematopoietic cell, a stem cell, or a BLS-2 cell. The cells may be administered back into the subject by intravenous, intramuscular, subcutaneous or intralesional routes.

Another embodiment of this invention is a method for the vaccination of a subject, which includes obtaining cells capable of MHC class II gene expression from the subject and transfecting them with a retroviral vector encoding CII-TA under suitable conditions so as to express CII-TA protein and induce MHC class II gene expression in the cells. The cells would then be tested for the presence of helper virus and other contaminants followed by the administration of clean cells to the subject to thereby induce expression of a class II human leukocyte antigen gene complex and thus vaccinate the subject. The cells obtained from the subject may be tumor cells, cells infected with a virus, or cells infected with a parasite. The cells may be administered back into the subject by intravenous, intramuscular, subcutaneous or intralesional routes.

The present invention provides a retroviral vector which includes a gene encoding CII-TA. The retroviral vector may be derived from a pGAG vector, a N2 vector, a SIM vector, a LNL6 vector, a LXSN vector or a MMuLV vector (Anderson, 1992; Miller, 1992; and Vande Woud et al., 1983; The contents of which are incorporated in their entirety into the subject application). The retroviral vector has a suitably modified 3' long terminal repeat and is replication incompetent. The vector may encode an adjuvant to the CII-TA protein activity such as the B7-1 or B7-2 protein. The present invention also provides a host cell transfected by the retroviral vector which encodes a gene encoding CII-TA protein. The host cell may be a virus packaging cell, a neoplastic cell, a somatic cell, a melanocyte, a hematopoietic cell, a stem cell, or a BLS-2 cell.

Several years ago, a BLS patient was shown to have the HLA class II negative defect that resulted in immunodeficiency. A cell line was derived from this patient who did not survive early childhood, and the resulting line was called BLS-2 (Hume et al, 1989; Hume and Lee, 1989). Examination

of the molecular characteristics of that cell line showed that all of the class II genes in BLS-2 were down regulated at the transcriptional level. The class II genes were intact and could be reexpressed by fusion with certain B cell lines. The defect, therefore, appeared to affect a *trans*-acting factor that was unlinked to the HLA region. Fusion experiments showed that cells derived from different patients could complement each other after transient fusion, demonstrating diverse genetic defects in cell lines derived from BLS patients (Hume and Lee, 1989; Seidl et al, 1992). A new patient who is a sibling of the original patient has been shown to lack the HLA class II molecules on all peripheral cells that have been examined.

A cDNA that complements the HLA class II negative regulatory defect in the mutant cell line, RJ2.2.5, was isolated recently (Steimle et al, 1993). The cDNA clone and gene were called CII-TA. The cell line BLS-2 which is a member of the same complementation group as RJ2.2.5 was transfected with the CII-TA cDNA and the defect in class II expression was complemented. Indeed, expression of the cDNA restores expression of HLA DR, DQ, and DP in BLS-2. Furthermore, a 72 nucleotide deletion of cDNA sequence in mRNA derived from BLS-2 was observed, and a splicing mutation in the corresponding gene was identified.

Several studies have begun to explore the function of CII-TA. First, when the CII-TA cDNA was introduced into class II-negative but γIFN inducible cells by transfection, cells that incorporated the vector became class II-positive (Steimle et al, 1995). In analyzing the kinetics of induction by γIFN of the CII-TA gene and class II genes, it was demonstrated that CII-TA expression is induced prior to the class II RNAs and that mRNA induction was not blocked by cycloheximide, whereas induction of class II mRNA is blocked by drug treatment. This suggested that protein synthesis was not required after addition of γIFN to activate

transcription of CII-TA. Thus, class II induction by γIFN appears to be mediated through CII-TA alone.

Although CII-TA was implicated as a transcription factor, it did not appear to bind DNA, and there was no homology with already characterized proteins to suggest that this was indeed its function. Two recent reports (Zhou and Glimcher, 1995; Riley et al, 1995) have now provided strong evidence that the CII-TA protein functions by interacting with transcription factors that bind to the proximal class II promoter, although the exact binding proteins that are involved remains unclear. First, it was shown that the N-terminal sequence of the protein contains an acidic activator region similar to those found in other transcription factors. The C-terminus appears to interact specifically with class II promoter binding proteins. Intriguingly, several alternatively spliced mRNAs were identified by cDNA cloning, although it appears that only two can give rise to different isoforms of the protein. Only the larger protein of the two, corresponding to the originally described coding region, is capable of complementing the transcriptional defect in the RJ2.2.5 mutant cell lines. It appears that the shorter protein is truncated at the C-terminus, leading to the possibility that the smaller CII-TA isoform might be involved in transactivation of other genes that contain some binding sites in common with class II promoters, and perhaps some different ones.

Patients with this HLA class II deficient congenital immunodeficiency have been notoriously resistant to complete engraftment after HLA mismatched bone marrow transplantation (Fischer and Griscelli, 1988). In addition, serious immune complications have already become manifest in the new patient (age 36 months), so gene therapy might be considered the best option for immune reconstitution. Several methods have been developed over the last decade for the

transduction of genes into mammalian cells for potential use in gene therapy. In addition to direct use of plasmid DNA to transfer genes, retroviruses, adenoviruses, parvoviruses, and herpesviruses have been used (Anderson et al., 1995; Mulligan, 1993; The contents of whch are incorporated in their entirety into the subject application). For transfer of genes into cells ex vivo and subsequent reintroduction into a host, as would be most feasible in immunodeficiency patients, retroviruses have been the vectors of choice. Advantages are that infection of retroviruses is highly efficient and that the provirus generated after infection integrates stably into the host DNA. A disadvantage however, is that stable integration requires cell division, and many of the earliest hematopoietic progenitor cells that would be the preferred targets of gene therapy, do not divide under conditions used for the infections and hence to not incorporate virus, or if they do they may not retain their potential to completely reconsitute a host. Notwithstanding this problem, it is possible that the long-term culture-initiating cells that can be transduced by retroviruses may be sufficient to repopulate some compartment with cells that are particularly long lived and stable.

Most current gene therapy protocols use murine retroviral vectors to deliver therapeutic genes into target cells; this process, which is called transduction, mimics the early events of retroviral infection. The crucial difference is that, unlike replication competent retroviruses, the vector genome packaged within the viral coat contains no genes for viral proteins and therefore is incapable of replication. The vector pGAG, used herein, is designed to have 3′ and 5′ long terminal repeat sequences necessary only for the integration of the viral DNA intermediate into the target host cell chromosome and a packaging signal that allows packaging into viral structural proteins supplied by the packaging line in *trans* (Miller, 1992; Wilson et al., 1990;

The contents of which are incorporated in their entirety into the subject application). Retroviral constructs were made in which the CII-TA cDNA was inserted downstream of the ß-actin promoter and the HLA class II associated invariant chain promoter to generate two different vectors. Genomic integration is the terminal step for these defective retroviral vectors. They cannot make viral proteins in cells transduced with the packaged vector and therefore cannot produce progeny virus. The CII-TA retroviral constructs were transfected into the virus packaging cell lines, AM12 and PG13, to generate infectious, but non-replicating virus particles. Cloning procedures and retroviral infection of cell lines are well known to one skilled in the art and detailed protocols may be found in Kriegler, 1990. Producer lines with high virus titers were chosen for these experiments for their ability to transduce the cell line BLS-2, resulting in HLA class II gene reexpression.

There are several protocols for human gene therapy which have been approved for use by the Recombinant DNA Advisory Committee (RAC) which conform to a general protocol of target cell infection and administration of transfected cells (see for example, Blaese, R.M., et al., 1990; Anderson, W. F., 1992; Culver, K.W. et al., 1991). In addition, U.S. Patent No. 5,399,346 (Anderson, W. F. et al., March 21, 1995, U.S. Serial No. 220,175) describes procedures for retroviral gene transfer. The contents of these support references are incorporated in their entirety into the subject application. Retroviral-mediated gene transfer requires target cells which are undergoing cell division in order to achieve stable integration hence, cells are collected from a subject often by removing blood or bone marrow. It may be necessary to select for a particular subpopulation of the originally harvested cells for use in the infection protocol. For example. white blood cells may be separated from red blood cells using an apheresis

procedure. The white cells may then be placed in culture with mitogens, such as IL-2 or OKT3 (which can stimulate T-cell proliferation) for approximately 18 hrs. Then, a retroviral vector containing the gene(s) of interest would be mixed into the culture medium. The vector binds to the surface of the subject's cells, enters the cells and inserts the gene of interest randomly into a chromosome. The gene of interest is now stably integrated and will remain in place and be passed to all of the daughter cells as the cells grow in number. The cells may be expanded in culture for a total of 9-10 days before reinfusion (Culver et al., 1991). As the length of time the target cells are left in culture increases, the possibility of contamination also increases, therefore a shorter protocol would be more beneficial. In addition, the currently reported transduction efficiency of 10-15% is well below the ideal transduction efficiency of 90-100% which would allow the elimination of the selection and expansion parts of the currently used protocols and reduce the opportunity for target cell contamination.

This invention provides for the construction of retrovirus vectors containing the cDNA for the transactivating factor CII-TA ultimately for use in gene therapy of Bare Lymphocyte Syndrome. The efficiency of transduction of these vectors can be tested in cell culture systems. In an initial effort to direct tissue restricted expression of the transduced CII-TA in infected cells, an internal promoter derived from the HLA class II associated invariant chain (Ii) gene was used in the retrovirus, but restricted expression was not obtained with this vector. The Ii promoter can be further characterized through the mutation of *cis*-acting elements that appear to be activated by CII-TA and the incorporation of them in a future series of vectors. The generation of ecotropic virus producer lines with selected vectors may be possible for correction of CII-TA defective mice recently developed.

This invention also provides for the infection of peripheral blood lymphocytes and hematopoietic precursor cells from bone marrow of a CII-TA defective BLS patient with the recombinant retroviruses. The efficiency of infection and tissue specificity can be assessed by flow cytometry. In experiments with bone marrow cells, the potential of short term culture systems to direct outgrowth of the stem cells along different pathways can be investigated. It is also important to assess the stability and longevity of these cells in vitro in long term cultures and in vivo by following their persistence and differentiation in Hu/SCID mice.

This invention provides for the analysis of the ability of CII-TA transduced cell lines from Bare Lymphocyte Syndrome patients to present specific antigens, superantigens and allo-antigens to appropriate HLA class II restricted T cell clones and to autologous and parental T cells. In particular, because it is possible that the patient's T cells developed in the absence of HLA class II molecules in the thymus, it was important to examine their responses to antigenic stimulation in vitro.

The invention described herein provides an unexpected improvement of transfection efficiency over the transfection efficiencies previously reported. The experimental details are more fully described in the following sections which provide a series of experiments to establish the efficacy of this therapeutic approach.

EXPERIMENTAL DETAILS

EXAMPLE 1: Initial experiments to establish feasibility of gene transfer in the BLS patient

As evidenced by the clinical data shown in Table I, the patient's responses to mitogens are relatively normal or

slightly depressed, whereas specific responses against fungal and bacterial antigens are extremely low or absent. Responses in pooled mixed lymphocyte cultures (MLC) were low, but natural killer cells were normal or perhaps slightly elevated. Thus, a profound immunodeficiency is exhibited.

Table I. Clincial laboratory data for BLS patient

		PATIENT	NORMAL RANGE
Mitogens:	unstimulated	271	50-1000
	PHA-P	174,353	169,410-331,750
	OKT3 (αCD3)	2114	3133-34,409
Antigens:	unstimulated	147	50-1000
	candida albicans	1055	27,795-178,954
	tetanus toxoid	104	500-42969
	staph enterotoxin B	15,700	56,961-136,039
	toxic shock syndrome toxin	329	53,233-124,467
Pooled MLC:	autostimulated	116	50-1000
	pooled MLC	16,565	18,778-102,740
Natural killer cell	100:1 50:1 25:1	84 90 43	15-72 15-72 15-72

A new cell line, BLS-2sib was established from peripheral B cells of the new patient. HLA typing showed that she shared only one HLA haplotype with her deceased sister (BLS-2). The specific region of the CII-TA gene known to contain a splice donor mutation in the patient's sister was amplified using PCR and the corresponding fragments were cloned in the vector TA (Invitrogen, San Diego). Sequencing of two independent clones showed that the same splice donor

mutation is found in the patient as in her sibling (Figure 1).

Many independent clones may be sequenced to insure a high probability that both alleles of the CII-TA gene from the patient have been sequenced. The patient's family is from Puerto Rico and although there appears to be no consanguinity in the family, it seems likely that both mutant alleles in the patient are identical. If there is doubt that the mutation is identical on both alleles, the PCR products can be sequenced directly, and the mutation on the second allele can be characterized if it is different by further mRNA analysis and PCR.

Vectors for gene therapy in BLS patients

A cDNA fragment for CII-TA was used for the generation of expression vectors. There are two goals to be met. First, to obtain efficient transfer of the gene into human hematopoietic cells, preferably stem cells. Second, because HLA class II gene expression is restricted to particular cell types and tissues, it would be beneficial to obtain some tissue specificity in the expression of the CII-TA and hence, HLA class II genes. Because of their high efficiency of infection and gene transfer, retroviruses are the transfer vectors of choice. The single promoter vector pGAG-ßactin could be useful because it contains a deletion in the 3' long terminal repeat (LTR) and upon integration into the recipient cell the LTR promoter is crippled by the deletion (Wilson et al, 1990). Because the transduction of such a vector containing the CII-TA gene can be monitored in the patient merely by analyzing cell surface expression of HLA class II molecules by flow cytometry, the efficiency of infection can be easily assessed. The disadvantage of this vector is that it does not contain a selectable marker and therefore provides no independent means of assuring that the vector and gene (cDNA) of interest has been taken up stably by the infected cell. However, selection for drug

resistance can interfere with expression of a second gene in a retrovirus vector through promoter competition and can also result in bystander death during the selection process. Therefore using a selectable marker frequently decreases expression or alters the specificity of expression for the gene of interest.

If the pGAG-ßactin vector is used, the ßactin promoter can be removed by restriction endonuclease digestion and replaced with a potentially tissue specific promoter of choice. Because the HLA class II associated invariant chain (Ii) is expressed with a distribution essentially identical to that of class II molecules, and because despite the defect in CII-TA in both cell lines derived from the BLS patients, the Ii gene was expressed, the Ii promoter was chosen as the ideal promoter to drive expression of CII-TA in this example. Primers were synthesized for PCR amplification of Ii promoter sequences to insert directionally into the pGAG vector from which the ßactin promoter had been excised using XhoI and BamHI.

A prepared fragment to which adapters containing BamHI compatible ends had been appended, was ligated into both the pGAG-ßactin and pGAG-Ii vectors to produce two recombinant vectors capable of expressing CII-TA (Figure 2). The plasmid, pGAG-ßactin-CII-TA/S, was deposited on June 7, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid pGAG-ßactin-CII-TA/S was accorded ATCC Accession Number 97211. The plasmid, pGAG-Ii-CII-TA/S, was deposited on June 7, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent

Procedure. Plasmid pGAG-Ii-CII-TA/S was accorded ATCC Accession Number 97212.

These vectors were cotransfected into AM12, a virus packaging cell line with SV2neo to generate infectious retrovirus. Packaging cell lines such as PA317 and others have been previously approved for human use (Rosenberg, et al., 1990). The efficiency of virus production by the AM12 cells was tested by taking supernatants from several clones that grew out and incubating them with BLS-2. Three days after infection the cells plus controls were assessed for the frequency of HLA class II (DR) expression. Efficiencies in the range of 0.4-0.7% positives were obtained using supernatants from bulk cultures and up to 1.2% for those derived from clones. Two clones from each vector producer line were selected for expansion.

Producer lines derived from the mouse ecotropic packaging line E86 have been generated via infection with a retroviral vector that carries CII-TA. The procedure used to generate a cell line that is capable of producing retroviral particles is well known to those skilled in the art. See PCT International Publication No. WO 92/05266 entitled "Packaging Cells" Jolley, D.J. et al., 1992 (The contents of this PCT publication is incorporated in its entirety into the subject application). Briefly, a cell line is generated which does not produce a replication-competent retrovirus but can provide the *trans*-acting factors required by a replication-defective retrovirus. Although the genome of the producer cell can express the *trans*-acting factors necessary to rescue a newly introduced defective viral genome, the genome of the producer cell cannot rescue itself. Retroviruses which are replicative-incompetent and express CII-TA protein may be used in the future to correct the defect in a transgenic CII-TA knock-out mouse that currently is being developed by other researchers.

The producer line, PG13, generates viruses with higher efficiencies of infection for primate hematopoietic cells because it uses an envelope gene from the Gibbon Ape Leukemia virus rather than the amphotropic virus counterpart (Miller et al, 1991). Producer clones for each construct with PG13 cells were established. The nonreplicative virus, PG13/GAG-Ii-CII-TA/S, was deposited on June 7, 1995 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The nonreplicative virus, PG13/GAG-Ii-CII-TA/S was accorded ATCC Accession Number 97212.

As before, titers for the clones were estimated by infecting BLS-2 cells with supernatants from the producer clones. Again, two clones that appeared to be producing the highest amounts of infectious virus were selected for expansion. Several vials of cells were frozen as soon as possible. Because the efficiencies of transduction of BLS-2 cells to HLA DR positive by virus preparations were still relatively low, different means of infecting cells were assessed. In one experiment, frozen virus supernatant, fresh virus supernatant, and co-cultivation of the PG13 virus producer cells with the BLS-2 cells were compared. The infected cells were stained with antibodies against HLA-DR and analyzed by flow cytometry. Table II shows the results of this experiment. As shown, a low efficiency of transduction by virus supernatants that had been collected and frozen at -80°C was observed, but the efficiency was somewhat increased by using supernatants that were collected and used immediately without freezing. However, the greatest efficiency was achieved by irradiating the virus producer cells with 3000 Rads and then co-cultivating them for 12 to 24 hr with the BLS-2 cells. In several more recent experiments the efficiency of transduction by co-cultivation has been in the range of 20 to 30% 72 hr post infection.

TABLE II. Efficiencies of transduction with retrovirus preparations.
(% HLA-DR positive)

Producer clone	pGAG-Ii/CII-TA#7	pGAG-Ii/CII-TA#2	no virus
Frozen virus supernatants	72 hr - 0.7 96 hr - 2.1	not done 1.1	0.1 0.1
Fresh virus supernatants	72 hr - 2.7 18 days - 11.1	not done	0.1
Co-cultivation with virus producers	72 hr - 12.5, 19.3	2.3	0.1

Therefore a culture of BLS-2 was followed that had been co-cultivated with PG13/pGAG-Ii/CII-TA virus producing cells, monitoring DR expression at 0, 3, and 8 days postinfection with no selection for expression. Figure 3 shows that the number of DR expressing cells gradually increases, suggesting either that the cells continue to integrate and express proviruses long after they are removed from the infecting cells, or that CII-TA gives the transduced cells a growth advantage. In addition, the expression of DR in the transduced cells is comparable quantitatively with that found on normal EBV transformed lines.

Lack of tissue specificity of the Ii promoter in pGAG-Ii.

Further experiments were performed in an effort to establish whether the modified vector with the Ii promoter replacing the ßactin promoter would result in tissue appropriate expression. In other words, when the CII-TA cDNA insert was placed downstream of the Ii promoter, it would be expressed only in cells that normally express the Ii chain gene.

Therefore, several nonlymphoid HLA class II and Ii negative cell lines were infected with the pGAG-Ii/CII-TA and pGAG-ßactin/CII-TA viruses. The results shown in Table III clearly show that there was no tissue specificity using the pGAG-Ii/CII-TA construct because all of the class II negative lines that were infected began to express HLA-DR within a few days of infection with viral supernatants.

Table III. HLA class II expression in nonlymphoid cells transduced with pGAG-Ii/CII-TA (% positive for HLA-DR)

Cell line	0 days post-infection	4 days p.i.	8 days p.i.
HeLa	0.1	37.0	72.1
U937	0.1	14.7	31.8
melanoma (DX-4)	0.1	20.4	84.8

Several more cell lines were evaluated after infection with this virus and all of them were able to respond by inducing their endogenous class II genes. Experiments using RNase protection of cellular RNA from the infected cells are in progress to ask whether the transcripts initiate at the Ii promoter or at the "defective" LTR promoter in the construct. This information should help in the development of a strategy to achieve tissue specific expression.

Infection of PBMCs and CD34+ BMCs from a BLS patient.

Co-cultivation of irradiated virus producing cells and the BLS-2 cells gives between 20 and 50% class II positive cells within a few days after infection in the absence of drug selection. These experiments suggest that the approach provided in this invention should be effective, and therefore, experiments were begun to transduce peripheral blood cells and bone marrow cells from the BLS patient.

First, the frequencies of T cell subsets and B cells were analyzed in the uninfected populations. The numbers of B cells and CD4+ T cells are greatly reduced. For example, less than 10% of T cells are CD4+.

Peripheral blood was obtained from the patient and the mononuclear cells were separated out with Ficoll-Hypaque. The cells were then infected overnight by co-cultivating them with irradiated PG13/pGAG-Ii/CII-TA producers in complete RPMI plus 15% FCS. Then the cells were removed and cultured in the presence of IL-2 and PHA. Although it is not the ultimate goal to induce the proliferation of T cells, this experiment was performed primarily to determine whether peripheral lymphocytes can be infected efficiently. Three days after infection approximately 6% of the cells were HLA class II positive. The frequency of expression in the cell population at 8 days postinfection was also assessed by double staining with CD3, CD20 (B cell marker), and HLA-DR. Most of the cells growing out in this system were T cells, positive for CD3, and again approximately 6% were also positive for HLA-DR (Figures 4A, 4B, 4C and 4D). Only a few were positive for CD20, but of these about half were also positive for HLA-DR. Although the numbers are very low, it is possible that some B cells were activated in this experiment.

Experiments are planned to culture the monocyte and T cell depleted lymphocytes from PBMCs in the presence of B cell stimulators such as Staphylococcus Protein A, anti-μ, and anti-CD40. These agents should induce proliferation of the peripheral B lymphocytes. Co-cultivation of stimulated cells with the virus producers will be performed and their ability to express DR after infection will be determined as in the above experiments.

MATERIALS AND METHODS

To construct retrovirus vectors containing the cDNA for the

transactivating factor CII-TA ultimately for use in gene therapy of Bare Lymphocyte Syndrome. To generate recombinant retroviruses for infection of murine cells to correct a CII-TA defect in knock-out mice recently developed.

Vector and producer characterization.

As described in the previous section, producer cell lines with AM12 and PG13 were generated with the pGAG-ßactin/CII-TA and pGAG-Ii/CII-TA constructs. This invention provides a protocol for highly efficient retroviral infection of the recipient cells. Since the ultimate goal will be to use this vector or one derived from it in gene therapy of the patient, the Ii promoter and CII-TA inserts that were introduced into the pGAG-ßactin vector will be sequenced, and considered for use in gene therapy by the Food and Drug Administration. Standard dideoxy sequencing protocols using primers generated in our laboratory from the known sequences will be used to confirm that no subtle mutations have arisen in the production of the CII-TA vector.

Secondly, it needs to be demonstrated that rearrangements do not occur frequently within the proviruses integrated in recipient cells. To show this genomic DNA can be isolated from the PG13/pGAG-Ii/CII-TA producer cells and from infected cell lines and PBMCs from the patient. The DNAs will be digested with appropriate restriction endonucleases that recognize sites within the virus, and analyzed by Southern blotting and hybridization with probes derived from the retrovirus backbone. If rearrangements are not occurring, patterns between the producer and recipient cells to be identical. These experiments will be performed with the PG13/pGAG-Ii/CII-TA vector. When new vectors derived from this one are generated, they will be analyzed in a similar manner.

CII-TA knock-out mice.

A homozygous CII-TA knock-out mouse has recently been derived. The mouse was engineered to delete the homologous murine exon to that deleted by the splicing mutation in the BLS patient. Class II antigens are absent on B cells, dendritic cells, and splenic macrophages, as expected. Further experiments are in progress to characterize these mice. Producer lines will be generated for infection of murine cells. When modified vectors are available, ecotropic or amphotropic producers from them will be generated.

Modification of the Ii promoter in generating further vectors.

Despite the use of a fragment of the Ii promoter to drive CII-TA expression it was not tissue specific in the context of the retrovirus vector and it could activate expression of class II genes in essentially all lymphoid and nonlymphoid cell lines tested and in a significant proportion of T lymphocytes from the patient. One of two possible mechanisms might account for the lack of tissue specificity of this construct. First, the fragment of the Ii promoter that we used is itself regulated by CII-TA (Chin et al, 1995), and leakiness in the uninduced promoter may be enhanced by expression of CII-TA as the major regulator of class II and Ii promoters. This coincides with the finding that the endogenous Ii gene is induced in all the cells that have been examined after transduction with the CII-TA containing viruses). Second, even if the Ii promoter itself is not leaky, the enhancer deletion in the 3' LTR of the vector construct may not be sufficient to inactivate it completely as a promoter after insertion of the infecting provirus into the recipient cell. These possibilities may be assessed by RNase mapping with RNAs isolated from infected cells (both BLS-2 and the transduced nonlymphoid cells that have been analyzed) using riboprobes derived from the LTR promoter of pGAG-ßactin and from the junction of Ii and CII-TA in the pGAG-Ii/CII-TA construct. A murine CD4

cDNA fragment will be inserted into pGAG-Ii to use as a control. (An appropriate control for the animal experiments will be human CD4 cDNA). If the expression of mCD4 from the Ii promoter is tissue specific, then it can be concluded that leakiness of either the LTR promoter or the Ii promoter is responsible for the initial expression of CII-TA in infected cells and that further enhancement of expression is due to the production of CII-TA itself.

If these experiments suggest that it is primarily the deleted LTR that is leaky, then generation of further deletions or mutations in the 3' LTR of the vector can be carried out. In order to do this, the promoter activity of the LTR and deletion mutants derived from it may be tested by inserting them into a reporter gene construct. To test for very small levels of expression a reporter such as luciferase is needed. Constructs would be transfected into Raji and Jurkat cells, both of which are easily transfected. Care will have to be taken not to delete sequences that will be necessary for processing at the 3' end of viral RNAs in the packaging cells.

On the other hand, if either the LTR or the Ii promoter is leaky in the experiments with the mCD4 control, reporter constructs may be generated with the Ii promoter and may be deleted or mutated at the putative regions that might interact with CII-TA. Initially, there was little information to suggest that the Ii gene itself my be regulated by CII-TA. But, two recent papers (Zhou and Glimcher, 1995; Riley et al, 1995) that have analyzed the role of CII-TA in transactivating class II promoters suggest that transactivation of the Ii promoter results from interactions between CII-TA and elements in the Ii promoter that are in common with class II promoters, such as the putative S and X boxes which are included in the 5' region of the Ii promoter fragment incorporated into pGAG-Ii/CII-TA (Brown et al, 1993). Thus, the first experiment is to

generate CAT reporter constructs with the full length Ii promoter fragment and one deleted for the S and X boxes. The ability of the two constructs to drive CAT expression will be compared when transfected into Raji, RJ2.2.5, and RJ2.2.5 cells infected with pGAG-Ii/CII-TA. The full promoter is expected to be more active in Raji and RJ2.2.5/CII-TA than it is in RJ2.2.5, hopefully the deleted Ii promoter will be equally active in all three cell lines. Since Ii is expressed at somewhat lower but reasonable levels in RJ2.2.5 and in BLS-2 cells (Long et al, 1984; Hume et al, 1989), this is likely. If so, a new retrovirus vector can be generated using the deleted Ii promoter. If not, other regions of the promoter can be examined by deletion or mutation as indicated in the literature to see if they are transactivated by CII-TA in the same manner (Barr and Saunders, 1991; Brown et al, 1994). These modified vectors will be tested to detect tissue specific expression by infecting class II positive and negative cell lines as already described.

<u>Infection of peripheral blood lymphocytes and hematopoietic stem cells of a CII-TA defective BLS patient with the recombinant retroviruses.</u>

In order to make an attempt at gene therapy in the patient, detectable expression of HLA class II antigens must be achieved following exposure of the patients cells to the retrovirus. Initial experiments shown in the previous section in which peripheral blood mononuclear cells and CD34+ bone marrow cells from the patient were infected suggest that this will be possible. Further experiments can now be directed to determine whether the expression can be directed into the appropriate cells by culturing under conditions that favor outgrowth of B cells, dendritic cells and macrophages. In these studies, the efficiency of transduction of CD34+ lineage negative cells will be assessed by examining qualitatively and quantitatively, the subsequent expression of HLA class II by differentiated

clonigenic precursor cells emerging following culture in selective media with defined cytokine support.

Long term bone marrow cultures will be performed according to the method of Gardner and Kaplan (1980) with minor modifications. The long term marrow culture medium will consist of supplemented McCoys medium containing 12.5% horse serum, 12.5 percent fetal calf serum and 1 micromolar hydrocortisone sodium succinate. At weekly intervals, half the cells will be removed from the long term cultures and analyzed for gene transfer and expression. Short term colony assays will be used to assess granulocyte-macrophage colony forming units (CFU-GM) according to a modification of the method of Pike and Robinson (1970). The dendritic cell precursors will be propagated from CD34+ lineage - cell fractions according to the method of Szabolcs *et al* (1995) as described herein.

When future vectors become available, their abilities to infect the CD34+ cells and to express class II in various subsets of cells will be analyzed. A substantial number of PBMCs and CD34+ bone marrow cells from the patient have been frozen for future experiments, although it will still be preferable to use freshly isolated cells if possible.

It is planned to stimulate the proliferation of B lymphocytes purified from PBMCs. The PBMCs are isolated from peripheral blood from a subject by Ficoll-Hypaque density gradient centrifugation and allowed to adhere to plastic tissue culture flasks for one hr at 37°C. Then the T cells are removed by E-rosetting. The enriched B lymphocytes can then be stimulated by crosslinking the B cell receptor (delivering signal 1) (Reth, 1992) or by triggering the CD40 receptor (signal 2) (Armitage and Alderson, 1995) in the presence of additional cytokines. In the first example, the cells can be cultured for one to three days in the presence of anti-immunoglobulin antibodies

or formalinized Staphylococcol Protein A-Cowan (Calbiochem) and IL-2. Then they can be co-cultivated with virus producing cells for 24 hrs and placed back into culture for several days and then evaluated for surface markers and class II expression. In the second example, the cells can be cultured either in the presence of anti-CD40 antibody and L cells expressing FcγII (to crosslink the antibody) or with cells expressing CD40 ligand and IL-4 and IL-2 (Banchereau et al, 1991; Armitage et al, 1993). B lymphocytes stimulated in this manner can proliferate for several weeks in culture. Again after a short period of stimulation, the B lymphocytes can be co-cultivated with the virus producing cells and placed back into cultures in which they can be evaluated for B cell markers such as CD19, CD20, CD80, CD86, etc. and HLA class II.

It is planned in all of these experiments to use cells from the BLS patient, since in most of these experiments there is no independent means of establishing whether cells are being infected when they are already expressing HLA class II. Indeed, it may not be possible to extrapolate from results using cells from a normal adult individual with a virus such as pGAG-Ii/mCD4 that we are constructing because of differences in lymphoid cell development between children and adults. Bone marrow and peripheral cells from the patient are stored frozen, in order to be able to proceed with some of these experiments in case the patient experiences complications that would not permit the collection of PBMCs and BMCs for a period of time. Using another virus is also problematic because it may not behave in a manner similar to pGAG-Ii/CII-TA.

A separate and designated area in the bone marrow transplant laboratory will be established where the virus producer cells can be grown and where infections and long term cultures can be done. Ultimately, it is possible to bank autologous serum from the patient in order to avoid

antigenic exposure of the cells by culturing in fetal calf serum and other additives.

Growth of CII-TA and unrelated vector transfected cells in SCID mice

In order to assess the long term viability and the expression of the pGAG-Ii/CII-TA transduced marrow cell fractions, peripheral blood T and B lymphocytes and dendritic cells, their growth may be evaluated in CB17 SCID mice that are sublethally irradiated (300 r) and treated with anti-Asialo GM1. Mosier *et al* (1990) have demonstrated that mature B and T lymphocytes will grow and can be detected for up to six months in the peripheral circulation of SCID mice following intraperitoneal injection. For these studies, inocula in excess of 10^7 cells will be administered intraperitoneally into the irradiated, anti Asialo GM1 treated mice. Thereafter, at 2, 4, 8, 12 and 16 weeks post infusion, groups of animals will be sacrificed and their blood, spleen and marrow samples analyzed for the presence of human T or B cells by immunohistochemical analyses as well as FACS analyses of mononuclear cell populations extracted from these tissues. The human cells will be co-stained with antibodies to HLA class II to ascertain the presence or absence of HLA class II expression.

In order to assess the long term expression of HLA class II on mature cells derived from myeloid progenitors, a modification of this model developed by Lapidot *et al* (1992) can be used in which similarly prepared mice are injected intraperitoneally with CD34 positively selected cells (Ceprate Columns, Cell Pro Corp) and then treated with the cytokines erythropoietin, SCF, IL-3 and GM-CSF. These animals can maintain production of human myeloid and erythroid cells for periods of at least 2 months after inoculation. The systems of McCune *et al* (1988) may be modified to examine whether marrow stromal fragments obtained by bone marrow biopsy from patients are able to

support the growth of hematopoietic cells following intraperitoneal injection into sublethally irradiated anti-Asialo GM1 treated SCID mice. This system would have the advantage that the stromal cell populations used to support the growth of hematopoietic cells would be derived from an HLA class II deficient autologous donor. Animals receiving these hematopoietic grafts will be assessed for circulating HLA class II positive macrophages and granulocytes and for CFU-GM and CFU-GEMM of human origin expressing HLA class II that can be differentially grown from the marrow, spleen and blood of these animals at biweekly intervals following intraperitoneal inoculation.

These studies of the long term expression of HLA class II are of prime importance for the assessments of the effects of *in vivo* growth on the expression of transfected vectors in and of marrow and lymphoid cell populations on the expression of the transfected vectors. In other gene transfer experiments, transfected and effectively integrated gene segments have been silenced such that expression can no longer be detected after variable periods of *in vivo* growth despite persistence of the integrated genes.

This next section will describe the analysis of the ability of CII-TA transduced and corrected cell lines from Bare Lymphocyte Syndrome patients to present superantigens, allo-antigens and specific antigens to appropriate HLA class II restricted T cell clones and to autologous and parental T cells.

Several strategies have been developed to test whether the immunogenicity of tumor cells can be increased through the transduction of CII-TA. In one model system, a murine class II negative melanoma cell line, B78H1 may be infected with viral particles which contain the recombinant CII-TA retroviral vector constructs produced by AM12 and E86 amphotropic and ecotropic producer cell lines. Vaccination

protocols would then be carried out in a mouse model. The efficacy of the vaccination may be assessed by quantitating the metastases to the lungs of the mouse after challenge with native tumor. Several other murine tumors are available for testing if the results prove to be encouraging. Whether the transduced human melanoma cells can effectively present peptide antigen to specific CD4+ T-cell clones is also an important yet testable question. Experiments to analyze antigen presentation by melanoma cells are underway.

Function of CII-TA corrected B cell lines.

The first goal in these experiments is to determine whether the CII-TA transduced into the patient's B cell line and the B cell line from her sibling, functionally complements not only the expression of class II antigens, but the ability of the cells to present antigen. Three distinct mechanisms will be evaluated. First will be the ability to present superantigens to peripheral T cells, then to present alloantigens to T cell clones and in MLR, and finally to present specific antigens to T cell clones that recognize the peptides restricted by the appropriate HLA-DR.

Recent studies have shown that the BLS-2 and 721.180 B cell lines supply adequate costimulation signals for T cell responses to superantigens even in the absence of class II (Dennig and O'Reilly, 1995). Superantigens, such as TSST-1 derived from a Staphylococcus exotoxin (toxic shock syndrome toxin 1) and SEB derived from Staphylococcoal enterotoxin B are known to bind to MHC class II positive antigen presenting cells (APCs), but are not processed through the intracellular compartment where the class II antigens normally associate with peptides (Herman et al, 1991). These class II superantigen structures can be recognized by the T cell receptor and induce oligoclonal T cell proliferation which is MHC unrestricted. Thus, large numbers of T cells can be activated by exposure to

superantigens plus APCs. It was shown that these superantigens do not bind to class II negative B cell lines or macrophages. Nevertheless, these B cell lines supply appropriate accessory signals for the proliferation of T cells selectively expressing vß3 or Vß2 TCRs when the T cells are stimulated with the superantigens SEB and TSST-1, respectively. The BLS patient's T cells could respond by generating T cells expressing the full array of Vß TCRs to superantigens if presented by class II positive lines.

In this invention, the ability of the BLS B cell lines and those transduced with CII-TA to bind SEB and TSST-1 will be assessed. To observe specific binding, the cells are first incubated with biotin conjugated superantigen with appropriate biotin labeled antibodies as positive and negative controls, then they are washed and stained with FITC-conjugated avidin. Only cells expressing class II should show detectable binding. These experiments will determine whether Vß2-expressing cells will proliferate in response to TSST-1 and Vß3 in response to SEB (as demonstrated in the absence of class II expression), or whether cells will expand more broadly to include Vß8 bearing TCRs, for example in response to TSST-1 and Vß2 in response to SEB. In these experiments, T cells are purified from peripheral blood of the donor and stimulated with TSST-1, SEB, or PHA and costimulatory cells treated with mitomycin C and irradiated as described (Dennig and O'Reilly, 1993) and restimulated every 2 or 3 days with either IL-2 or superantigen. After 2 weeks, the T cells are analyzed by flow cytometry. Since there was no detectable difference in the pattern of expansion that correlated with the absence of class II in the BLS patient as the T cell donor, T cells obtained from normal donors can be used. If processing and transport of class II molecules in the CII-TA corrected cells are normal, a response similar to control class II positive B cells should be observed.

Another test for appropriate functioning of the class II molecules on CII-TA corrected cells will be to determine whether they can stimulate T cells in a mixed lymphocyte culture (MLC). The uninfected and PG13/pGAG-Ii/CII-TA infected B cell lines from the patient can be irradiated and used as stimulator cells. They are allowed to incubate with an equal number of PBMCs from various HLA compatible and incompatible donors in the presence of RPMI plus 10% human serum. After 4 days in culture, the cells are pulsed overnight with ^{3}H-thymidine, and the next day they are harvested and incorporation is measured by scintillation counting. In addition to MLCs, several allospecific T cell clones recognizing the DRw13 molecule (DRB1 1301), the allele that the patient carries on both haplotypes, were generated for the Tenth International HLA Workshop (Mickelson et al, 1989). They could be used in a stimulation assay as well. In either type of experiment, the HLA mismatched T cells should proliferate in response to the transduced cell but not in response to the class II negative untransduced cell. Proliferation should not occur in response to the untransduced cell by autologous T lymphocytes, but should occur in response to the transduced cell. This might give an indication of whether any selection of T cells during development has occurred in the patient.

It would also be interesting to determine whether antigen specific T cell clones restricted by the patient's DRB1 1301 molecule and known peptide (for antigens such as influenza hemagglutinin or tetanus toxoid) would be able to recognize the patients transduced cell plus peptide or whole antigen. If such clones can be identified, an attempt will be made to obtain them and to proceed with the experiment.

Function of BLS patient's T cells.

Because HLA class II molecules have not been detected in the peripheral blood mononuclear cells or any other tissues of

the BLS patient, it is plausible that the development of the patient's T cells has occurred in the absence of class II and thus has not been subject to negative or positive selection. This could lead to a situation in which autoreactive CD4+ T cells are present in the periphery and potentially could be activated when class II positive APCs are introduced into the patient. It might be difficult to identify specific autoreactive T cell clones if only small numbers of the patient's CII-TA corrected APCs are available for stimulation of her T cells, but a model system has been developed which should be appropriate to ask this question.

The development of Epstein Barr Virus induced lymphoproliferative disease (EBV-LPD) in bone marrow transplant recipients has been studied and correlated the onset of EBV-LPD with the severe deficiency of EBV-specific cytotoxic T cells during the first six months post-transplant. Sensitive methods have been developed based on an earlier report (Bourgault et al, 1991) to detect both CD8+ cytotoxic T lymphocyte and CD4+ helper lymphocyte precursors from peripheral blood. Briefly, PBMCs isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation are allowed to adhere to plastic tissue culture flasks for one hr at 37^0C. Adherent monocytes and macrophages are harvested and subsequently irradiated and used as feeder cells. The monocyte depleted PBLs are then incubated with monoclonal antibodies followed by immunomagnetic beads to remove CD20+ and CD56+ cells. Bulk cultures are then set up with the purified T cells and irradiated adherent cells, and autologous B-LCLs. They are cultured for 12 days in conditioned medium containing IL-2. Limiting dilution cultures are set up in an analogous fashion. To assay cytotoxic T-lymphocyte precursor frequencies, limiting dilution culture wells are mixed with ^{51}chromium labelled target cells (in this case the B-LCLs). Helper/inducer T cells can be detected in proliferation assays in which parallel limiting dilution cultures are

incubated with irradiated B-LCLs and ^{3}H-thymidine incorporation is measured. Because the patient is homozygous in the class II region, the CTLp and THp frequencies can be compared within her T cell population in response to her own class II negative B-LCL and her CII-TA corrected B-LCL, with those of her parents, although allospecific CTLp may be detected in the cultures with parental T cells. This should not detect EBV specific CTLp in the patient's peripheral T cells regardless of the presence of CII-TA and hence class II, but it should in those of the parents. This would indicate that the patient has not been able to generate at anti-EBV cytotoxic response. However, it is not known whether specific THp cells are present.

If on the one hand, negative selection in the thymus is the primary mechanism to limit the expansion of autoreactive cells, then some cells may be detected growing out that are essentially autoreactive or EBV specific in response to her own CII-TA corrected B-LCL but not in response to her uncorrected B-LCL. On the other hand, if there is little evidence for the proliferation of TH cells after exposure to class II, then expansion of autoreactive cells may not be a significant problem.

The present invention will involve the use of human subjects. Participation in these studies by human subjects will involve the drawing of peripheral blood samples and bone marrow for isolation of mononuclear cells. Risks to the participants are the minimal risks associated with venipuncture and the risk of local anesthesia for bone marrow aspiration as described in the attached consent forms. Informed consent will be obtained from all participants.

CB-17 SCID-SCID mice sublethally irradiated (300r) and treated with anti-Asialo GM1, will serve as the model for

the experiments proposed. Animals to be used will be 8-12 week old females. The animals will receive intraperitoneal injections of marrow cell fractions, mature T, B and dendritic cells or BLCL transduced with the CIITA vector or a nonsense vector. The animals will be assayed at 0.5, 1, 2, 4 and 6 months for evidence of persistent engraftment of human cells in different lineages and their expression of HLA Class II. Animals will be sacrificed in groups at defined intervals post-transplant to assess the origins of the cells detected in the tissues.

The SCID mouse has been chosen because of the results of detailed studies indicating that this animal, after treatment with anti-mouse Asialo-GMI, with or without irradiation, permits the continuous growth of fresh human T and B lymphocytes BLCL and, if supported with cytokines or stroma, marrow derived cells. These animals congenitally lack defined T- and B-cell function. After treatment with ∝ Asialo-GMI, they also lack NK cell mediated graft resistance.

During the course of these experiments, the animals will be housed in microisolator cages under specific pathogen free conditions and will receive autoclaved food, water and prophylactic Bactrim. The animals will be divided into experimental groups in single microisolator cages at 5 animals/cage. The animals will be observed daily and specific assessments of weight and general condition will be obtained on a weekly basis.

The procedures to be used involve the minimal pain of inoculation under sterile conditions. The animals are inoculated within a comfortable tube restraining device. At the time of euthanasia, animals are sacrificed by cervical dislocation.

Method of Vaccination

Because Bare Lymphocyte Syndrome is an extremely rare congenital defect, only a small number of patients will potentially benefit directly from the gene transfer studies we propose. However, this syndrome can be considered a good model system in which to ask questions about the feasibility of gene transfer into early hematopoietic precursors, the ability to regulate which subsets of cells ultimately express the gene of interest, the numbers of cells required to restore immune reactivity to the lymphoid compartment, the importance of thymic selection after restoration of MHC class II expression, and many others. For these reasons, we assert that the experiments proposed will have significance for many other diseases in which gene therapy might be considered.

Additionally, the unexpectedly high transduction efficiency may allow one to harvest neoplastic cells from a tumor in a subject, transduce the tumor cells in culture as described above and administer the transduced cells back to the subject as a vaccination against further neoplasia. The high levels of HLA-DR gene expression produced in the tumor cell lines suggest this possibility of developing a system in which cells cultivated directly from fresh tumors could be induced to express their endogenous class II genes. This may render the tumor cells more immunogenic *in vivo*. If endogenous HLA class II genes could be expressed on cells derived from a neuroblastoma, a treatment for this particularly aggressive pediatric cancer may result. As a first step in this process, the immunogenicity of the transduced neuroblastoma cells could be assessed in a Hu/SCID mouse. SCID mice lack a functional immune system, however a rudimentary immune system can be developed in them by transfer of hematopoietic stem cells derived from normal bone marrow. It may be possible to develop an immune response in SCID mice through the expression of CII-TA.

The results described above support the efficacy of a gene

therapy protocol which consists of a vaccination strategy for tumors in the treatment of human neoplastic conditions and infections which result from abnormal gene expression disorders.

Example 2: CORRECTION OF DEFECTIVE EXPRESSION IN HLA CLASS II DEFICIENCY (BARE LYMPHOCYTE SYNDROME) CELLS BY RETROVIRAL TRANSDUCTION OF CIITA

Retrovirus mediated gene transfer was used to restore HLA class II expression to HLA class II negative patient cells from complementation group A(II) of HLA class II immunodeficiency or Bare Lymphocyte Syndrome (BLS). A vector, pGAG/Ii-CIITA, was constructed with the HLA class II associated invariant chain promoter driving CIITA expression. Co-cultivation with the virus producer line was consistently shown to be the optimal method for infection of all cell types. The induction of MHC class II expression after virus infection was rapid, and high levels of expression were achieved within one week of infection. It is that a small quantity of CIITA expressed from the LTR promoter activates the Ii promotor within the vector and sets up a self propelling system. Together, these characteristics should prevent promoter repression and provide stable, though not tissue specific expression. Transduced B-lymphoblastoid cell lines readily established tumors in CB17-*scid/scid* mice and the HLA class II positive cells demonstrated a clear competitive advantage *in vivo*. Ultimately, use this transduction system may be used to restore normal immune function to a BLS patient for which no other therapeutic option currently exists.

Immune responses to protein antigens require T lymphocyte activation and differentiation following recognition of peptides bound and presented at the cell surface by major histocompatibility complex (MHC) class I and II molecules. MHC class II molecules present peptides to CD4+ T

lymphocytes, amplifying the regulatory arm of the T cell response (helper T cells). Lack of expression of the human MHC or HLA class II molecules results in a lethal immunodeficiency called Bare Lymphocyte Syndrome (BLS) (5,6), which is characterized by an absence of T cell responses and impaired antibody production leaving patients susceptible to viral, bacterial, and fungal infections (7).

Genes encoding the HLA class II α:ß heterodimers (DP, DQ, and DR) are located in a segment of several hundred kilobases (kb) on human chromosome six (8). These molecules are expressed constitutively on "professional" antigen presenting cells (APCs) including B cells, macrophages, and Langerhans-dendritic cells of the skin and lymphoid organs (1). They are also expressed transiently along the developmental pathway of many hematopoietic cell types or following stimulation with soluble factors, such as interferon-γ, TNF-α, and IL-4 (1-4). Most HLA class II positive cells express the DR, DP and DQ α and ß genes coordinately suggesting that a common mechanism regulates their expression. Genetic evidence supporting this hypothesis has come from extensive studies of mutant B-cell lines generated in vitro or established from BLS patients (9-15). These cell lines show a concomitant decrease in expression of all HLA class II antigens and all appear to affect transcription of the corresponding genes (7,16-19). In the few families which have been studied by pedigree analysis, BLS is inherited as an autosomal recessive trait, unlinked to the HLA region (20). Studies using somatic cell fusion and heterokaryon analyses provided support for the recessive nature of the defects (21-25). Furthermore, there are established at least four and possibly five complementation groups for the mutations affecting HLA class II transcription.

A B lymphoblastoid cell line, BLS-2, has been established from a BLS patient who did not survive early childhood (19).

All of the HLA class II genes in BLS-2 were down regulated at the transcriptional level. The class II genes, however, were intact and could be reexpressed by fusion with certain B cell lines (21). The defect, therefore, appeared to affect a trans-acting factor that was unlinked to the HLA region. Experiments showed that cells derived from different patients could complement each other after transient fusion, demonstrating diverse genetic defects in cell lines derived from BLS patients. Recently, a sibling of the original patient has been diagnosed with BLS and shown to lack the HLA class II molecules on all peripheral cells that have been examined. A regulatory cDNA, called CIITA, that complements the HLA class II transcriptional defect in complementation group II (A) which includes BLS-2 has now been cloned (26). Although not a DNA binding protein itself, CIITA is thought to associate with the transcription complex via proteins bound to several of the upstream cis-acting elements (27,28). Expression of the CIITA cDNA in BLS-2 restores expression of HLA DR, DQ, and DP. Indeed, a 75 nucleotide deletion of cDNA sequence in mRNA derived from BLS-2 was observed, and a splicing mutation in the corresponding gene was identified (26).

Because patients with BLS have been notoriously resistant to complete engraftment after bone marrow transplantation and the prognosis for HLA mismatched marrow transplants is dismal in these patients (29), gene therapy might be considered the best option for immune reconstitution. Here we present the development of a retrovirus vector containing CIITA for gene therapy of BLS.

Materials and Methods

Cell lines and reagents. All lymphoblastoid cell lines were grown in RPMI 1640 (Gibco-BRL Lifesciences) supplemented with 15% fetal calf serum (purchased from Hyclone, Logan, Utah), L-glutamine, essential and non-essential amino acids, sodium pyruvate (all purchased from Gibco). Adherent

cell lines were grown in Dulbecco's modified Eagle Medium (DMEM) with high glucose containing the same supplements as above, but without sodium pyruvate. All cell lines were grown at 37°C in 5 % CO_2/air. The EBV-LCL BLS-2sib was established by infection of PBL from the patient with supernatants from the EBV-secreting marmoset cell line, B95-8, and was propagated in complete medium as above but with penicillin, streptomycin, and anti-PPLO agent (all Gibco).

Unconjugated and fluorescein or phycoerythrin conjugated antibodies for HLA-DR, CD3, CD20, and CD86 were purchased from Becton-Dickenson (San Jose, CA) and used according to manufacturer's protocols.

Construction of retrovirus vectors and establishment of producer lines. pGAG/ßactin (30) were chosen to construct the vector. The ßactin promoter was removed by digestion with XhoI and BamHI and replaced it with a PCR generated fragment from the HLA class II associated invariant chain (Ii) promoter. Ii is expressed with a distribution essentially identical to that of class II molecules, and despite their defect in HLA class II expression cell lines derived from CIITA deficient BLS patients express Ii (19). Oligonucleotides used to prime the reaction were: sense-5' GGGGAGACAACTCGAGGTTGTCTTCTGTTTCAAAGT (SEQ ID NO 3) and antisense-5' GACGGATCCTGCTTCTCCTCCTGTGTCATCTGGG (SEQ ID NO 4).

After the reaction, the product was precipitated and digested with XhoI and BamHI, and ligated into the deleted pGAG vector. The resulting construct was designated pGAG/Ii. A cDNA fragment for CIITA with BamHI-SalI adaptors (sense strand 5'GATCCGAAGGGGTTCG (SEQ ID NO 5) and antisense strand 5'TCGACGAACCCCTTCG) (SEQ ID NO 6) was ligated into BamHI digested pGAG/Ii and pGAG/ßactin. The resulting constructs were designated pGAG/Ii-CIITA and pGAG/ßactin-CIITA.

Infection and co-cultivation. Supernatants were collected from subconfluent monolayers of virus producing cells after 8-16 hrs at 37°C and used directly or frozen at -80C. Target cells were incubated with the supernatants in the presence of 4 μg/ml polybrene in complete RPMI plus 15% fetal calf serum for 4 -6 hr at 37°C. The cells were washed and resuspended in RPMI at a concentration of 0.5 x 10^6/ml and cultured at 37°C. Alternatively, producer cells were irradiated with 3000 Rads in a gamma irradiator and then co-cultivated with 5 x 10^6 cells per T25 flask for 24 hr.

Flow cytometry. Fluorescinated or phycoerythrin conjugated antibodies were purchased from Becton Dickinson and used for staining as recommended by the manufacturer. The cells were analyzed on an FACScan fluorescence-activated cell sorter (Becton-Dickenson, San Jose, CA).

RNase protection assays. The construct for generating an RNA probe from the LTR region was made by inserting a HincII-PstI fragment from the 5'LTR and gag region of pGAG/Ii-CIITA into pGEM3Z (Promega, Madison, WI). Fragments of DNA should be protected by RNA originating from the LTR promoter. The construct from the Ii-CIITA junction of pGAG/Ii-CIITA was made by inserting a XhoI-NcoI fragment from the 5' end of the Ii promoter fragment to the NcoI site in CIITA into pGEMblue. A cluster of fragments should be protected by RNA initiating at the Ii promoter, whereas a fragment should be protected by RNA from the actin promoter from pGAG/ßactin-CIITA infected cells. Endogenous CIITA RNA should protect a fragment. ^{32}P labelled RNA probes were generated by transcription of the linearized templates with SP6 RNA polymerases (Ambion, TX) in conditions recommended by the manufacturer. Probes were purified by electrophoresis through denaturing polyacrylamide gels and eluted by extraction with RNAzol. Total RNAs were hybridized and products analyzed using the RPAII kit from Ambion.

Isolation and cultivation of peripheral blood mononuclear cells. Using standard sterile conditions, fifteen ml of whole blood was collected from the BLS patient and ficolled using Histopaque 1077 (Sigma, St. Louis, MO). A total of 70 x 10^6 peripheral blood mononuclear cells were isolated. 3 x 10^6 cells were resuspended in medium supplemented with IL-2 and PHA as previously described (31). pGAG/Ii-CIITA PG13 producers or untransfected PG13 cells that had been seeded at a concentration of 4 x 10^5 cells per 6 well dish 24 hr prior to blood collection, were irradiated as described above, and 10^6 PBMCs were added to each well. The cells were co-cultivated for 24 hr at 37°C. Non-adherent cells were removed from the producers and placed back into the incubator at 37°C. The cells were analyzed by flow cytometry 6 days post infection.

Xenotransplantation of EBV cell lines into CB-17/scid/scid mice. 5-8 week old CB-17/*scid/scid* mice purchased from Taconic Farm (Germantown, NY) were maintained in microisolater cages (Thoren Cagen, NY), with 3 to 5 mice per cage under specific pathogen free conditions. The mice were treated intraperitoneally with 30 μl rabbit anti-asialo GM1 antiserum (α-asialo GM1) (Wako Chemicals, Richmond, VA) on days -1, 8, and every 5 to 8 days thereafter for the duration of the study for depletion of endogenous NK cell function. The animals received subcutaneous injections of B-LCLs resuspended in Matrigel ® (Collaborative Biomedical Products, Dedford, MA) at 10-20 x 10^6 cells per mouse. The cells were either untransduced or transduced with the pGAG/Ii-CIITA vector. The animals were monitored over the course of twelve weeks for tumor development and sacrificed at twelve weeks when their tumors were excised. Single cell suspensions were prepared from the tumors and analyzed by flow cytometry. Five mice were injected per cell line.

Results

Vectors for gene therapy in BLS patients

A new cell line, BLS-2sib was established from peripheral B

cells of the new patient. The specific region of the CIITA gene, at the exon-intron boundary known to contain a splice donor mutation in BLS-2, was amplified using PCR and the corresponding fragment was cloned in the vector TA (Invitrogen, San Diego). Sequencing of 7 independent clones showed that the identical mutation was found in the BLS-2sib line, and supports the notion that the mutated allele is homozygous.

In generating a vector for the expression of CIITA, two goals were paramount. First, to obtain efficient transfer of the gene into human hematopoietic cells, preferably stem cells. Second, because HLA class II gene expression is restricted to particular cell types and tissues, to obtain tissue specificity in the expression of the CIITA and hence, HLA class II genes was the objective. Because of their high efficiency of infection and gene transfer, retroviruses were used. The single promoter vector pGAG/ßactin was chosen because it contained a deletion in the 3' long terminal repeat (LTR) and upon integration into the recipient cell the LTR promoter would be crippled by the deletion (30). Because the transduction of such a vector containing the CIITA gene was monitored merely by analyzing cell surface expression of HLA class II molecules by flow cytometry, it seemed possible to assess the efficiency of infection easily (32). The disadvantage of this vector is that it does not contain a selectable marker and therefore provides no independent means of assuring that the vector and gene (cDNA) of interest has been taken up stably by the infected cell. However, selection for drug resistance can interfere with expression of a second gene in a retrovirus vector through promoter competition (33) and can also result in bystander death during the selection process. Therefore using a selectable marker may decrease expression or alter the specificity of expression for the gene of interest.

If the pGAG/ßactin vector was used, the ßactin promoter

could be removed by restriction endonuclease digestion and replaced with a tissue specific promoter of choice. Because the HLA class II associated invariant chain (Ii) is expressed with a distribution essentially identical to that of class II molecules, and because, despite the defect in CIITA in cell lines derived from the BLS patients, the Ii gene was expressed, it was decided that the Ii promoter should be used as the ideal promoter to drive expression of CIITA in the vector.

A prepared fragment of CIITA cDNA with SalI-BamHI adaptors was ligated into both the pGAG/ßactin and pGAG/Ii vectors to produce two recombinant vectors capable of expressing CIITA (Figure 2). These vectors were cotransfected into AM12 and PG13 packaging cell lines with SV2neo to generate infectious retrovirus (34,35). The producer line, PG13, generates viruses with high tropism for primate hematopoietic cells because it uses an envelope gene from the Gibbon Ape Leukemia virus. The efficiency of virus production by the producer cells was tested by taking filtered supernatants from several clones that grew out and incubating them with BLS-2. Three days after infection, the cells plus controls were assessed for the frequency of HLA class II (DR) expression. Efficiencies in the range of 0.4-0.7% were obtained using supernatants from bulk cultures and up to 1.2% for those derived from clones. Two clones from each vector producer line were selected for expansion.

Because the efficiencies of transduction of BLS-2 cells to HLA DR positive by virus preparations were still relatively low, an assessment of different means of infecting cells was begun. In one experiment, frozen virus supernatant, fresh virus supernatant, and co-cultivation of the PG13 virus producer cells with the BLS-2 cells were compared. The infected cells were stained with antibodies against HLA-DR and analyzed by flow cytometry. Table I shows the results of this experiment. It was observed that a low efficiency

of transduction by virus supernatants that had been collected and frozen at -80C, and the efficiency was somewhat increased by using supernatants that were collected and used immediately without freezing. However, the greatest efficiency was achieved by irradiating the virus producer cells with 3000 Rads and then co-cultivating them for 12 to 24 hr with the BLS-2 cells. In several more recent experiments the efficiency of transduction by co-cultivation has been in the range of 20 to 30% 72 hr post infection. Figure 5 shows the expression of HLA-DR on BLS-2 cells which had been cocultivated with the pGAG/Ii-CIITA PG13 virus producers. The cells were analyzed at 0, 3, and 8 days postinfection with no selection for expression. To generate a control vector, the murine CD4 cDNA was inserted into pGAG/ßactin and monitored transduction by staining with a monoclonal antibody that recognizes CD4. The cells showed expression of murine CD4, but an absence of HLA-DR, confirming the conclusion that CIITA was in fact inducing class II expression. Expression of cDNAs other than CIITA in pGAG/Ii was very low or undetectable after transduction.

pGAG/Ii-CIITA is not tissue specific.

Further experiments were performed to establish whether the modified vector with the Ii promoter replacing the ßactin promoter would result in appropriate tissue expression. It was possible that CIITA would be expressed only when the provirus integrated in cells that normally express the Ii chain gene. Therefore, several nonlymphoid HLA class II and Ii negative cell lines were infected with the pGAG/Ii-CIITA and pGAG/ßactin-CIITA viruses. The results clearly indicated that tissue specificity had not been achieved, because all of the class II negative lines that were infected began to express HLA-DR within a few days of infection with viral supernatants as shown in Table II. These results were extended to cell lines derived from several different types of tissues including T cells, many different carcinomas, as well as neuroblastomas and

teratocarcinomas that are not inducible for HLA class II expression. Furthermore, normal human fibroblasts and explants from fresh tumor specimens have been transduced using this virus. In every case, at least a portion of the transduced cells expressed HLA DR whether or not the cells are capable of expressing HLA class II after induction with interferon-γ. Table VI lists all of the cell lines and types that have been successfully transduced with pGAG/Ii-CIITA.

The expression of both HLA-DR and Ii in these cells were then examined by Northern blotting experiments. Both DRA and Ii mRNA were induced *de novo* by the expression of CIITA after introduction of the vectors. The induction of Ii by CIITA confirms an earlier report (36). Experiments using RNase protection of cellular RNA from the infected cells indicate that despite the deletion in its enhancer, the LTR promoter functions quite efficiently (Figures 4A and 4D). In this experiment, it was observed that ample quantities of RNA that initiated at the 5' LTR promoter in HeLa cells infected with either pGAG/ßactin-CIITA or pGAG/Ii-CIITA, although the LTR was less efficient in expression in the BLS-2 lymphoblastoid cell line. When a probe derived from the Ii promoter/CIITA junction was used in a similar experiment, again high levels of RNA were detected arising from the internal promoter, a set of three bands designated Ii/CIITA in Figures 4C and 4F. RNA arising from the ßactin promoter is designated ßactin/CIITA, although that could also represent readthrough from the LTR promoter. Intriguingly, in all of the transduced cells, another band that migrates at the same position as the endogenous CIITA RNA in BLS-2 is found (endogenous CIITA). It is not found in untransduced HeLa or DX-4 cells. This implies that CIITA may also activate it own promoter. Thus, it is possible that transcription from the LTR promoter first generates CIITA, and subsequently the CIITA activates transcription from the Ii promoter in the construct and from the

endogenous promoter in the transduced cells. These properties of pGAG/Ii-CIITA should allow highly stable and persistent expression of MHC class II in transduced cells.

pGAG/Ii-CIITA can transduce peripheral blood cells efficiently

Co-cultivation of irradiated virus producing cells and the BLS-2 cells gives between 30 and 50% class II positive cells within a few days after infection in the absence of drug selection. These experiments suggested that this approach will be effective, and therefore, experiments were begun to transduce peripheral blood cells and bone marrow cells from the patient. Peripheral blood mononuclear cells (PBMCs) were obtained from the patient, the mononuclear cells separated with Ficoll-Hypaque, and were infected by co-cultivating them with irradiated pGAG-Ii/CIITA PG13 producers. Then the PBMCs were removed and cultured in the presence of IL-2 and PHA. Three days after infection approximately 6% of the cells were HLA class II positive (Figure 5). Since retroviruses integrate only in actively dividing cells (37) and a large proportion of PBMCs are terminally differentiated and not dividing, this frequency of transduction was encouraging. The cell population at 8 days postinfection was analyzed by double staining with CD3, CD20 (B cell marker), and HLA-DR. Most of the cells growing out in this system were T cells, positive for CD3, and again approximately 6% were also positive for HLA-DR. Only a few were positive for CD20, but of these about half were also positive for HLA-DR. Although the numbers are low, it appears that some B cells expressing CD20 were transduced and activated in this experiment.

pGAG/Ii-CIITA is stably expressed both in vitro and in vivo.

Although the CIITA transduced BLS-2 cell line were cultured *in vitro* for several months and they remained HLA class II positive, it was unclear whether the expression would be stable *in vivo*. To address this question, transduced and

untransduced BLS-2 cells were injected into CB17-*scid/scid* mice. The CB17-*scid/scid* mouse was chosen because detailed studies indicate that this animal, after treatment with anti-mouse Asialo-GMI, with or without irradiation, permits the continuous growth of fresh human T and B lymphocytes, and B-LCL(38). These animals congenitally lack defined T and B cell function, and after treatment with ∝ Asialo-GMI, they also lack NK cell mediated graft resistance. The transduced BLS-2 line was >90% HLA-DR positive after immunomagnetic sorting for DR expression. In addition, transduced BLS-2sib cells that were approximately 12% HLA-DR positive at the time of injection and untransduced BLS-2sib cells were injected. Lymphomas developed in the mice over a period of twelve weeks. Figures 7A, 7B, 7C and 7D show that transduced HLA class II positive tumors had a slightly higher rate of tumor growth than those derived from untransduced cells, although more cell lines will have to be assessed in these mice before concluding that HLA class II expression affects the ability of cells to establish tumors. When the tumors were excised at twelve weeks and stained with anti-HLA-DR antibodies, those that were not transduced remained class II negative, while the transduced BLS-2 cells remained highly positive. Remarkably, however, one tumor derived from BLS-2sib cells was 92% HLA-DR positive in contrast to those maintained *in vitro* which remained about 10-15% positive during the course of the experiment. Tumors from the other mice of this group were transferred to new mice and remained highly HLA-DR positive. Thus, CIITA and hence class II expression appears to confer a competitive advantage to the cells for growth *in vivo*.

Discussion

Over the last decade the possibility of introducing genes to correct inherited defects or to impart new functions to cells has entered research and clinical arenas. Many investigators have demonstrated efficient and stable expression of specific genes using various vector systems

and methods of transduction. Herein is described the use of retroviral mediated gene transfer to restore HLA class II expression to HLA class II negative cell lines and patient cells from complementation group II of Bare Lymphocyte syndrome. Ultimately, the goal is to restore normal immune function to patients with an ultimately fatal condition for which no other therapeutic option currently exists. The retroviral delivery system that we chose is advantageous because it is possible to achieve stable introduction of the gene of interest into the host chromosome allowing for long term expression in the cell and its progeny. It has been consistantly observed that co-cultivation with the pGAG/Ii-CIITA producer line, is the optimal method for infection of all cell types transfected.

CIITA, the MHC class II trans-acting regulatory factor, is necessary for class II transcription and expression (26,32). Expression of the wild type CIITA gene in BLS-2 cells drives HLA class II expression. Experiments infecting HLA class II negative nonlymphoid cells verified the essential link that CIITA plays in turning on HLA class II (32,39). The expression of this regulatory factor activated HLA class II expression in all cells tested, even those normally not inducible by gamma interferon or other means.

Experiments to analyze promoter specificity suggest that the CIITA within the vector drives itself. As well as transactivating HLA class II expression, CIITA also transactivates invariant chain expression (36). Figures 4A-4F show that both the LTR and Ii promoters in pGAG/Ii-CIITA are functional in different types of cells. It is possible that the CIITA expressed from the LTR promoter in nonlymphoid cells activates the Ii promotor and sets up a self propelling system. Based on the data, it is also possible that CIITA activates its own endogenous promotor in the transduced cells. Together, these characteristics of the vector system should prevent potential promoter

repression as has been described for retroviral vectors (40).

A vector with a drug selection marker was not used because of a concern that a drug resistance gene operating under the control of one promoter would interfere with expression of the gene of interest from a second promoter (33). As shown in Table II, drug selection for cells transduced with pGAG/Ii-CIITA is indeed unnecessary. In most cases integration is complete within 24 to 48 hours after infection (41), and expression of HLA class II molecules follows CIITA expression within a few hours (32). Thus, by 72 hours after co-cultivation, the vast majority of infected cells should be HLA-DR positive. Because it has been shown that dramatic increases in HLA-DR expression of several lines, particularly HeLa and a melanoma line, DX-4, between day 3 and day 8 post-infection, it is possible that the transduced cells compete favorably *in vitro* with uninfected HLA class II negative cells. In the case of BLS-2 and BLS-2sib, though the expression was low the cells could be further selected by sorting with anti-HLA DR antibody. More remarkably, transduced BLS-2sib cells after introduction into CB17-*scid/scid* mice showed a clear competitive advantage *in vivo*. The line that was only 12% positive at the time of injection, induced a tumor that was 92% positive at twelve weeks (Figure 7). Subsequent experiments in which the tumor cells were transferred to new animals demonstrated again that the HLA class II expression is stable. A small increase in the growth rate of the tumors from transduced cells compared with that for untransduced cells was observed during the experiment (Figures 7A-7D). That the difference in growth of the class II positive and negative tumors was immunologically mediated in this xenotransplantation system in mice without T cells is unlikely. Experiments are currently in progress to determine if it is the introduction and activation of wild type CIITA in the cell or HLA class II expression itself which plays a role in the advantage

these cells clearly exhibit over the uninfected class II negative cells. These results confirm the prediction that expression should be very stable and suggest further that the CIITA transduced cells may have a competitive advantage in a BLS patient. Indeed, if there is a competitive advantage for the CIITA transduced bone marrow progenitors in BLS patients, then engraftment of the transduced cells will be selected.

The work clearly shows correction of the genetic defect in HLA class II expression for BLS-2 and BLS-2sib. When PBMCs from the patient were transduced, stimulating with PHA and IL-2, mainly T cell expansion occurred. Figure 6 shows that, a small percentage of these T cells became HLA class II positive. However, it was also observed that a small number of CD20+ cells existed in the culture. It was intriguing that even in conditions not particularly favorable for B cell proliferation, a majority of this B cell population may have been activated and transduced. Nonetheless, the data show that CIITA is expressed in virtually every type of cell that has been infected with pGAG/Ii-CIITA. This contrasts sharply with the very tight regulation of CIITA and HLA class II expression in normal individuals. If this vector is ultimately used for gene therapy, it is possible that broad expression in all hematopoietic tissues could have deleterious effects, for example, activating potentially autoreactive T lymphocytes or altering developmental signals. On the other hand, it is not clear that restricted expression, though theoretically desirable, is necessary. Evidence from studies of T cell activation suggests that cells expressing MHC class II molecules in the absence of appropriate costimulatory molecules (such as B7-CD80 or B7.2- CD86 (42)) do not trigger naive T cells to proliferate, and may induce T cell death or anergy. Thus, inappropriate class II expression on cells that do not bear costimulatory molecules is not likely to result in activation of autoresponsive T cells and thus

should not lead to autoimmune complications in patients. A strategy that has been chosen to circumvent possible problems encountered because of unrestricted expression is to use specific cytokines direct the differentiation of transduced hematopoietic progenitors *in vivo* into the appropriate lineages that normally express HLA class II before returning the cells to the patient.

Examination of the functional properties the genetically manipulated cells from BLS patients exhibit is underway, and it has been demonstrated that these class II positive, transduced cells elicit a normal allogeneic response, and present superantigens and specific antigens to T cells. Ultimately the goal is to use pGAG/Ii-CIITA with appropriate transduction and culture systems to develop a practical therapy that can be applied to treat these patients. In addition, the vector we describe can be used in a wide variety of human and murine cells to induce stable MHC class II expression to understand the contribution of class II genes in many immunologic disease models.

TABLE IV. Efficiencies of transduction with retrovirus preparations.

Producer clone	pGAG-Ii/CIITA#7	pGAG-Ii/CIITA#2	no virus
Frozen virus supernatants	72 hr - 0.7% 96 hr - 2.1%	not done 1.1%	0.1% 0.1%
Fresh virus supernatants	72 hr - 2.7% 18 days - 11.1%	not done	0.1%
Co-cultivation with virus producers	72 hr - 12.5%, 19.3%	2.3%	0.1%

Table V. HLA class II expression in nonlymphoid cells transduced with pGAG-Ii/CIITA (% positive for HLA-DR)

Cell line	0 days post-infection	4 days p.i.	8 days p.i.
HeLa	0.1	37.0	72.1
U937	0.1	14.7	31.8
melanoma (DX-4)	0.1	20.4	84.8

Table VI. Cell types transduced with pGAG/Ii-CIITA with positive expression of MHC class II.

Hematopoietic	Non-hematopoietic
Human BLS-2 and BLS-2sib (B lymphoblastoid), PBMCs, U937 (monocyte), Jurkat (T lymphocyte), BLS CD34+ bone marrow cells	DX-4 (melanoma) HeLa (epithelial carcinoma) MCF-7 (breast carcinoma) LAN-1, NMB-7 (neuroblastoma) Tera2 (teratocarcinoma) BeWo (choriocarcinoma) normal and BLS derived bone marrow stroma
Murine EL-4	NIH 3T3, L cells (fibroblast) B78H1 (melanoma)

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Example 3: GENE THERAPY IN CIITA DEFICIENT BARE LYMPHOCYTE SYNDROME

The defective gene for one complementation group of HLA class II immunodeficiency or Bare Lymphocyte Syndrome has been identified as the transactivator, CIITA. Using recombinant retroviruses, it is possible to activate class II expression in the appropriate cells and to establish the feasibility of inserting and expressing the vector in peripheral lymphocytes and/or bone marrow derived cells from BLS patients. Cell lines have been genertaed that produce CIITA containing viruses in a modified vector and developed methods for highly efficient transduction of the virus in cell line. The efficiencies of transduction was compared using different methods with the cell line BLS-2.

The infected cells were stained with antibodies against HLA-DR and analyzed by flow cytometry. The greatest efficiency was achieved by irradiating the virus producer cells and then co-cultivating them for 12 to 24 hr with the target cells. The efficiency of transduction by co-cultivation has been in the range of 20 to 30% 72 hr post infection. Although it was hoped to achieve tissue specificity using the class II associated invariant chain (Ii) promoter, it was observed that all class II negative cell lines that were infected with this vector, could be induced to express class II. Furthermore, there is evidence that leaky expression of CIITA from either promoter can up-regulate the Ii promoter and drive high levels of class II expression.

PBMCs from a BLS patient were infected with the vector, and subsequently cultured in the presence of IL-2 and PHA. Three days after infection most of the cells growing out in this system were T cells, and approximately 6% were positive for HLA-DR. A few were positive for the CD20 B cel l marker, but of these about half were also positive for HLA-DR, suggesting that B cells might be preferentially transduced. CD34+ cells were obtained and cocultured with the producers in the presence of FCS and cytokines. Following coculture, the expanding cells were harvested and recultured with *c-kit* ligand, GM-CSF, and TNFα. The myeloid progeny were successfully transduced, based on HLA-DR expression by 29% of the bulk population. Moreover, among the population expressing CD1a after one week of expansion, 37% of these CD1a positive dendritic/Langerhans cells expressed HLA-DR compared to the control.

In order to assess stability *in vivo*, SCID mice were injected with transduced and untransduced B cells from BLS patients. The tumors were established over the course of 12 weeks. There was a small increase in the growth rate of tumors from transduced cells. The transduced BLS cells that were uniformly positive in culture remained at least 85%

positive after the lymphoma developed, and a line that had a low percentage of positive cells increased its expression dramatically. These results confirm that expression should be very stable and also suggest that the CIITA transduced cells may have a competitive advantage *in vivo*. Indeed, if there is a competitive advantage for the CIITA transduced bone marrow progenitors in the BLS patient, then engraftment of the transduced cells will be selected after introduction.

GENE THERAPY IN CIITA DEFICIENT BARE LYMPHOCYTE SYNDROME

One group HLA class II negative immunodeficiency or Bare Lymphocyte Syndrome (BLS), is due to a defect in the transactivator, CIITA. Using recombinant CIITA retroviruses, B cell lines have been transduced and primary cells from BLS patients and stained the cells with antibodies against HLA-DR. Irradiating virus producer cells and then co-cultivating with target cells achieved efficiencies of 20 to 50% 72 hr post infection. It has been observed that all class II negative cell lines that have been infected with this vector, can be induced to express HLA class II. CD34+ cells were obtained and cocultured with the producers in the presence of FCS and cytokines. Expanding cells were harvested and cultured with c-*kit* ligand, GM-CSF, and TNFα. The myeloid progeny were successfully transduced, based on HLA-DR expression by 29% of the bulk population. Moreover, after one week of expansion, 37% of CD1a positive dendritic/Langerhans cells expressed HLA-DR. Cells from the patient have been restored to functional competence in their abilities to stimulate in MLR, present superantigens, and specific antigens.

GENE THERAPY IN CIITA DEFICIENT BARE LYMPHOCYTE SYNDROME

HLA class II negative Bare Lymphocyte Syndrome, BLS, is an autosomal recessive disorder due to an inability to transcribe HLA class II genes. Because the products of these genes are crucial factors in the specific immune response, BLS patients are unable to generate antibodies and

T cells against a wide variety of pathogens and ultimately succumb early in life to infections. The defective gene for one complementation group of BLS has now been identified as the HLA class II transactivator, CIITA. Using recombinant retroviruses, we hope to activate class II expression in the appropriate cells and to establish the feasibility and efficiency of inserting and expressing the vector in peripheral lymphocytes and/or bone marrow derived cells from BLS patients. Cell lines have been generated that produce CIITA containing viruses in a modified vector and developed methods for highly efficient transduction of the virus in cell lines.

The efficiencies of transduction of frozen virus supernatant, was compared with fresh virus supernatant, and co-cultivation of virus producer cells with an established B cell line from a BLS patient. The infected cells were stained with antibodies against HLA-DR and analyzed by flow cytometry. The greatest efficiency was achieved by irradiating the virus producer cells and then co-cultivating them for 12 to 24 hr with the target cells. The efficiency of transduction by co-cultivation has been in the range of 20 to 30% 72 hr post infection. Although it was hoped to achieve tissue specificity using the class II associated invariant chain (Ii) promoter, it was observed that all class II negative cell lines infected with this vector, can be induced to express class II. Furthermore, there is evidence that leaky expression of CIITA from either promoter can up-regulate the Ii promoter and drive high levels of class II expression.

PBMCs from the patient were infected with the vector, and subsequently cultured in the presence of IL-2 and PHA. As expected, three days after infection most of the cells growing out in this system were T cells, and approximately 6% were positive for HLA-DR. A few were positive for the CD20 B cel l marker, but of these about half were also

positive for HLA-DR, suggesting that B cells might be preferentially transduced. Finally, CD34+ cells were obtained and cocultured with the producers in the presence of FCS and cytokines. Following coculture, the expanding cells were harvested and recultured with c-*kit* ligand, GM-CSF, and TNFα. The myeloid progeny were successfully transduced, based on HLA-DR expression by 29% of the bulk population. Moreover, among the population expressing CD1a after one week of expansion, 37% of these CD1a positive dendritic/Langerhans cells expressed HLA-DR compared to the control.

BLS is a good model system in which to ask about the feasibility of gene transfer into early hematopoietic precursors, targeting subsets of cells that will express the gene of interest, the numbers of cells required to restore immune reactivity to the lymphoid compartment, the importance of thymic selection after restoration of MHC class II expression, and many others. The results will have significance for many other diseases in which gene therapy might be considered.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Lee, Janet S.

(ii) TITLE OF INVENTION: RETROVIRUS VECTORS FOR EXPRESSION OF CII-TA AND ACTIVATION OF HLA CLASS II GENE EXPRESSION AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Cooper & Dunham LLP
(B) STREET: 1185 Avenue of the Americas
(C) CITY: New York
(D) STATE: New York
(E) COUNTRY: U.S.A.
(F) ZIP: 10036

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: White, John P
(B) REGISTRATION NUMBER: 28,678
(C) REFERENCE/DOCKET NUMBER: 47480-A-PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 212-278-0400
(B) TELEFAX: 212-391-0525

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGTTTGCGT AAGCAA 16

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAGTTTGCAT AAGCAA 16

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GACGGATCCT GCTTCTCCTC CTGTGTCATC TGGG 34

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATCCGAAGG GGTTCG 16

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TCGACGAACC CCTTCG 16

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What is claimed is:

1. A method to induce MHC Class II gene expression in a cell capable of MHC Class II gene expression which comprises transfecting the cell with a retroviral vector encoding a CII-TA protein under suitable conditions so as to express the CII-TA protein and induce MHC Class II gene expression.

2. The method of claim 1, wherein the retroviral vector is a pGAG DNA retroviral vector, a N2 retroviral vector, a SIM retroviral vector, a LNL6 vector, a LXSN vector or a MMuLV retroviral vector.

3. The method of claim 1, wherein the retroviral vector has a suitably modified 3' long terminal repeat which is replicative incompetent.

4. The method of claim 1, wherein the retroviral vector comprises a tissue specific promoter.

5. The method of claim 4, wherein the promoter is an HLA Class II associated invariant chain promoter.

6. The method of claim 1, wherein the retroviral vector comprises DNA in a 3' to 5' direction encoding the reverse transcript of at least a portion of a retrovirus, including a 3' long terminal repeat sequence, a promoter sequence, a sequence encoding a CII-TA protein and a 5' long terminal repeat sequence.

7. The method of claim 1, wherein the suitable conditions for transfecting the cell are co-cultivating a target cell with a virus packaging cell which comprises a retroviral vector encoding a CII-TA protein.

8. The method of claim 7, wherein the virus packaging cell is ψCRIP, ψCRE, AM12, E86, PA317 or PG13.

9. The method of claim 1, which further comprises introducing into the cell a suitable immunoenhancer or adjuvant.

10. The method of claim 9, wherein the adjuvant is B7-1 protein or B7-2 protein.

11. The method of claim 9, wherein the adjuvant is transfected into the cell on a retroviral vector encoding only the adjuvant.

12. The method of claim 9, wherein the adjuvant is introduced to the cell by co-transfection which comprises linking a sequence encoding the adjuvant to the retroviral vector encoding CII-TA protein.

13. The method of claim 1, wherein the cell is a neoplastic cell, a somatic cell, a melanocyte, a hematopoietic cell, a stem cell, or a BLS-2 cell.

14. A method to stimulate a specific, high-level T-cell response in a subject, which comprises

(a) obtaining cell(s) capable of MHC Class II gene expression from the subject;

(b) transfecting the cell(s) from step (a) with a retroviral vector encoding CII-TA under suitable conditions so as to express CII-TA protein and induce MHC Class II gene expression in the cell(s); and

(c) administering the cell(s) from step (b) to the subject to thereby induce expression of a Class

II human leukocyte antigen gene complex and thus stimulate the specific, high-level T-cell response in the subject.

15. The method of claim 14, wherein the subject has Bare Lymphocyte Syndrome or a neoplastic condition.

16. The method of claim 14, wherein the retroviral vector is a pGAG DNA retroviral vector, a N2 retroviral vector, a SIM retroviral vector, a LNL6 vector, a LXSN vector or a MMuLV retroviral vector.

17. The method of claim 14, wherein the retroviral vector has a suitably modified 3' long terminal repeat which is replicative incompetent.

18. The method of claim 14, wherein the retroviral vector comprises a tissue specific promoter, an HLA Class II associated invariant chain promoter or a ß-actin promoter.

19. The method of claim 14, wherein the retroviral vector comprises DNA in a 3' to 5' direction encoding the reverse transcript of at least a portion of a retrovirus, including a 3' long terminal repeat sequence, a promoter sequence, a sequence encoding a CII-TA protein and a 5' long terminal repeat sequence.

20. The method of claim 14, wherein the suitable conditions for transfecting the cell are co-cultivating a target cell with a virus packaging cell which comprises a retroviral vector encoding a CII-TA protein.

21. The method of claim 14, wherein the suitable conditions for transducing the cell are co-cultivating a target cell with PG13/GAG-Ii-CII-TA/S nonreplicative virus.

22. The method of claim 20, wherein the virus packaging cell is ψCRIP, ψCRE, AM12, E86, PA317 or PG13.

23. The method of claim 14, which further comprises introducing into the cell a suitable immunoenhancer or adjuvant.

24. The method of claim 23, wherein the adjuvant is B7-1 protein or B7-2 protein.

25. The method of claim 23, wherein the adjuvant is transfected into the cell on a retroviral vector encoding only the adjuvant.

26. The method of claim 23, wherein the adjuvant is introduced to the cell by co-transfection which comprises linking a sequence encoding the adjuvant to the retroviral vector encoding CII-TA protein.

27. The method of claim 14, wherein the cell is a neoplastic cell, a somatic cell, a melanocyte, a hematopoietic cell, a stem cell, or a BLS-2 cell.

28. The method of claim 14, wherein the cells are administered by intravenous, intramuscular, subcutaneous or intralesional routes.

29. A method for the vaccination of a subject, which comprises

(a) obtaining cell(s) capable of MHC Class II gene expression from the subject;

(b) transfecting the cell(s) from step (a) with a retroviral vector encoding CII-TA under suitable conditions so as to express CII-TA protein and induce MHC Class II gene expression in the

cell(s); and

(c) administering the cell(s) from step (b) to the subject to thereby induce expression of a Class II human leukocyte antigen gene complex and thus vaccinate the subject.

30. The method of claim 29, wherein the cell from step (a) comprises a somatic cell, a tumor cell, a cell infected with a virus, and a cell infected with a parasite.

31. The method of claim 29, wherein the retroviral vector is a pGAG DNA retroviral vector, a N2 retroviral vector, a SIM retroviral vector, a LNL6 vector, a LXSN vector or a MMuLV retroviral vector.

32. The method of claim 29, wherein the retroviral vector has a suitably modified 3' long terminal repeat which is replicative incompetent.

33. The method of claim 29, wherein the retroviral vector comprises a tissue specific promoter.

34. The method of claim 33, wherein the promoter is an HLA Class II associated invariant chain promoter.

35. The method of claim 29, wherein the retroviral vector comprises DNA in a 3' to 5' direction encoding the reverse transcript of at least a portion of a retrovirus, including a 3' long terminal repeat sequence, a promoter sequence, a sequence encoding a CII-TA protein and a 5' long terminal repeat sequence.

36. The method of claim 29, wherein the suitable conditions for transfecting the cell are co-cultivating a target cell with a virus packaging cell which comprises a retroviral vector encoding a CII-TA protein.

37. The method of claim 36, wherein the virus packaging cell is ψCRIP, ψCRE, AM12, E86, PA317 or PG13.

38. The method of claim 29, which further comprises introducing into the cell a suitable immunoenhancer or adjuvant.

39. The method of claim 38, wherein the adjuvant is B7-1 protein or B7-2 protein.

40. The method of claim 38, wherein the adjuvant is transfected into the cell on a retroviral vector encoding only the adjuvant.

41. The method of claim 38, wherein the adjuvant is introduced to the cell by co-transfection which comprises linking a sequence encoding the adjuvant to the retroviral vector encoding CII-TA protein.

42. The method of claim 29, wherein the cell is a neoplastic cell, a somatic cell, a melanocyte, a hematopoietic cell, a stem cell, or a BLS-2 cell.

43. The method of claim 29, wherein the cells are administered by intravenous, intramuscular, subcutaneous or intralesional routes.

44. A retroviral vector which comprises a gene encoding CII-TA.

45. The retroviral vector of claim 44, which comprises pGAG-ßactin-CII-TA/S or pGAG-Ii-CII-TA/S.

46. The retroviral vector of claim 44, wherein the retroviral vector may comprise a pGAG vector, a N2 vector, a SIM vector, and a MMuLV vector.

47. The retroviral vector of claim 44, wherein the retroviral vector has a suitably modified 3' long terminal repeat which is replicative incompetent.

48. The retroviral vector of claim 44, wherein the vector encodes an adjuvant to the CII-TA protein activity.

49. The retroviral vector of claim 48, wherein the adjuvant is B7-1 and B7-2 protein.

50. A host cell transfected by the retroviral vector of claim 44.

51. The host cell of claim 50, wherein the host cell comprises a virus packaging cell.

52. The host cell of claim 50, wherein the host cell is a neoplastic cell, a somatic cell, a melanocyte, a hematopoietic cell, a stem cell, or a BLS-2 cell.

FIGURE 1

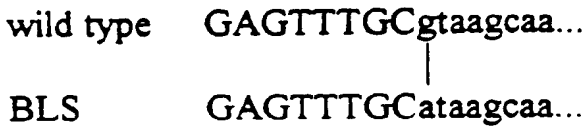


FIGURE 2

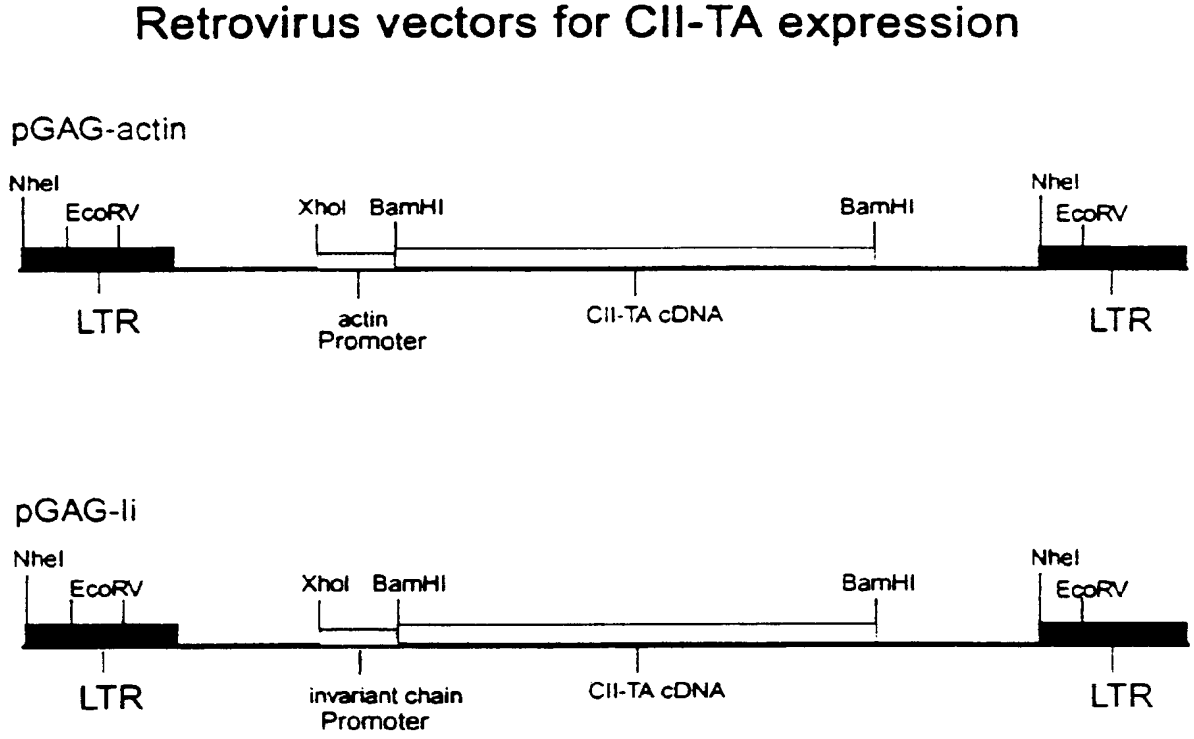

Retrovirus vectors for CII-TA expression
pGAG-actin
NheI
EcoRV
XhoI
BamHI
BamHI
NheI
EcoRV
LTR
actin Promoter
CII-TA cDNA
LTR
pGAG-Ii
NheI
EcoRV
XhoI
BamHI
BamHI
NheI
EcoRV
LTR
invariant chain Promoter
CII-TA cDNA
LTR

FIGURE 3A

Uninfected

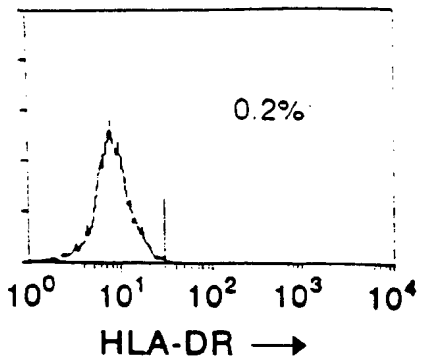


FIGURE 3B

3 days p.i.

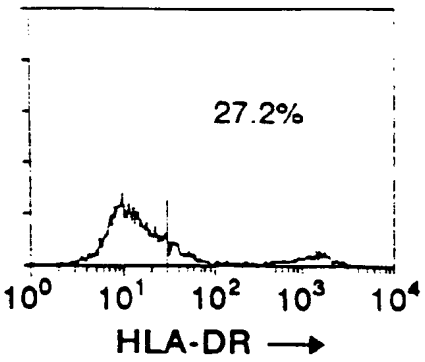


FIGURE 3C

8 days p.i.

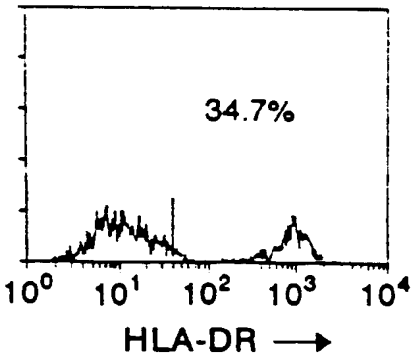


FIGURE 3D

Raji (+ control)

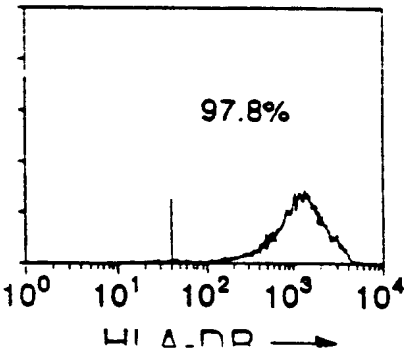


FIGURE 4A

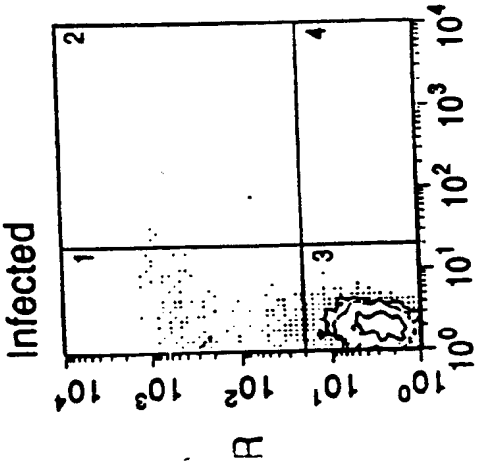


FIGURE 4B

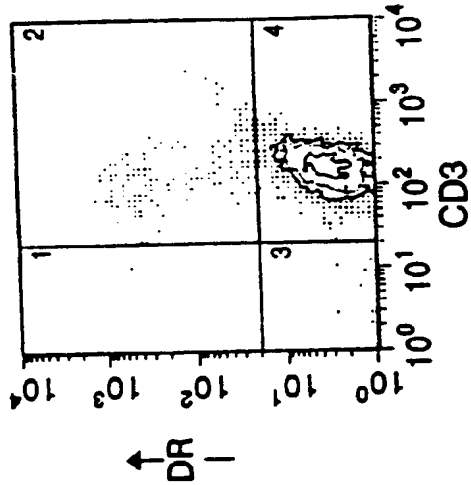


FIGURE 4C

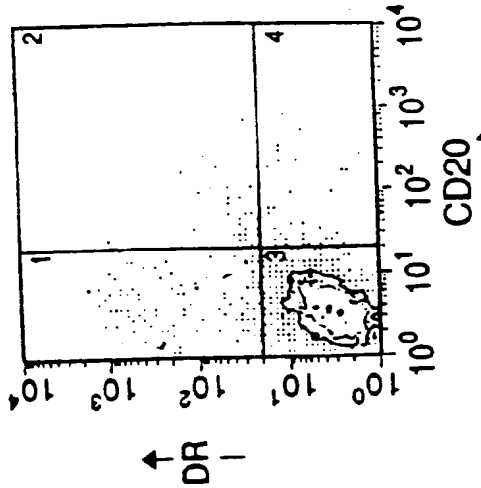


FIGURE 4D

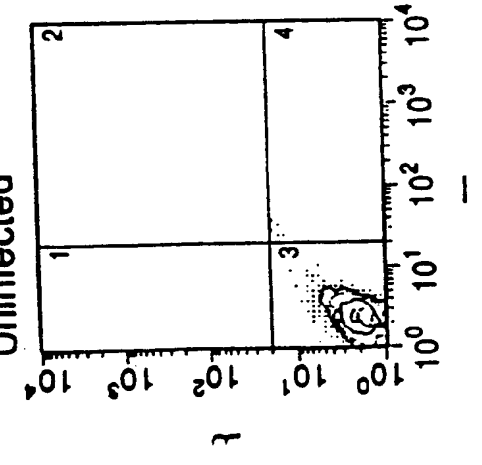


FIGURE 4E

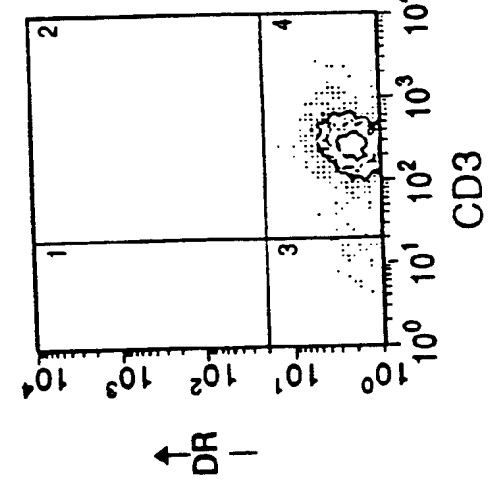


FIGURE 4F

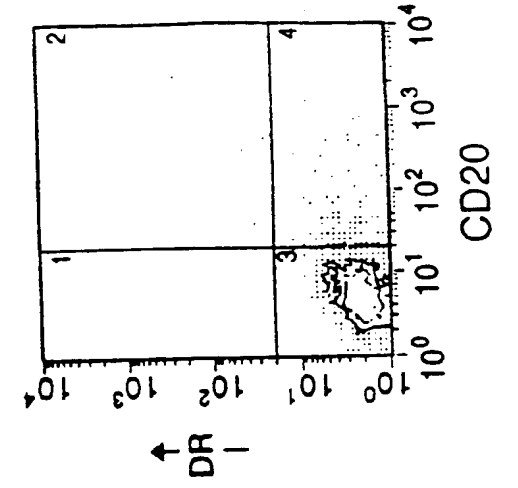


FIGURE 5

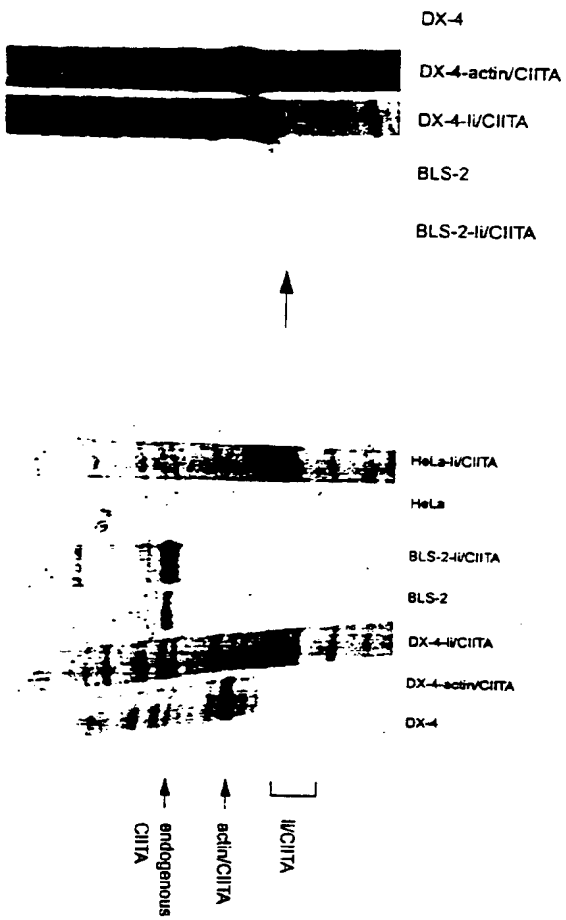

DX-4
DX-4-actin/CIITA
DX-4-Ii/CIITA
BLS-2
BLS-2-Ii/CIITA
HeLa-Ii/CIITA
HeLa
BLS-2-Ii/CIITA
BLS-2
DX-4-Ii/CIITA
DX-4-actin/CIITA
DX-4
endogenous CIITA
actin/CIITA
Ii/CIITA

FIGURE 6

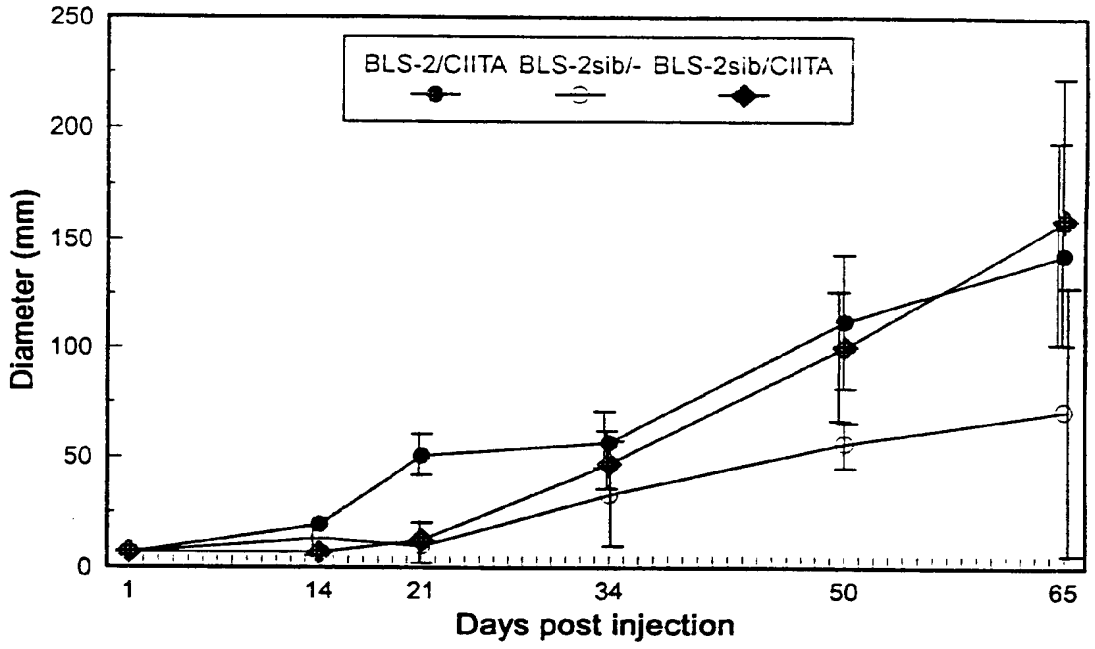

BLS-2/CIITA
BLS-2sib/-
BLS-2sib/CIITA
Diameter (mm)
250
200
150
100
50
0
1
14
21
34
50
65
Days post injection

FIGURE 7A

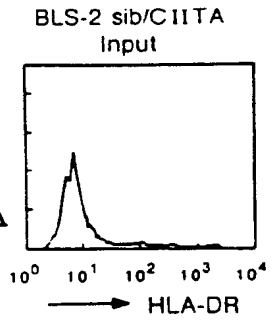


FIGURE 7C

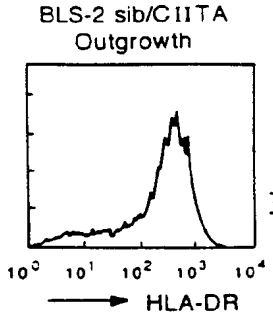


FIGURE 7B

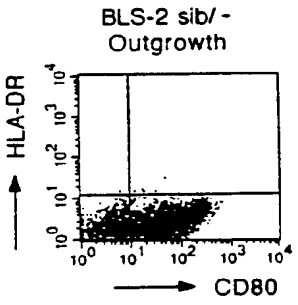
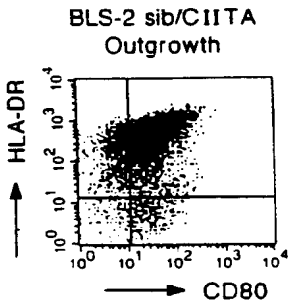


FIGURE 7D



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08044

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 38/16, 48/00, 49/00; C12N 15/00, 15/10, 7/00, 7/01,15/86
US CL :435/69.1, 172.3, 320.1; 424/93.6, 185.1, 287.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.3, 320.1; 424/93.6, 185.1, 287.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Biosis, Medline, Biotech
Search terms: MHC Class II, retrovirus vector, vaccine, gene therapy, CII-TA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,399,346 A (W.F. ANDERSON ET AL.) 21 March 1995, columns 5-6 and Claims 9-14.	1-52
A	BASKAR et al. MHC class II-transfected tumor cells induce long-term tumor-specific immunity in autologous mice. Cellular Immunology. 1994, Vol. 155, pages 123-133, especially pages 131-132.	1-52
A	DANOS et al. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proceedings of the National Academy of Sciences. September 1988, Vol. 85, pages 6460-6464, especially Figures 1-3.	1-52

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 21 JULY 1996	Date of mailing of the international search report 14 AUG 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID GUZO Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*